UNDENATURED VIRUS-FREE BIOLOGICALLY ACTIVE PROTEIN DERIVATIVES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to undenatured virus-free biologically active protein-containing compositions. More especially, this invention relates to the inactivation of viruses, especially lipid coated viruses, e.g., hepatitis B in human blood, blood component, blood plasma or any fraction, concentrate or derivative thereof containing blood proteins non-blood sources including normal or cancer cells, the exudate from cancer or normal cells grown in culture, hybridomas, in products from gene splicing (DNA), etc., by use of di- or trialkyl phosphates, and to the resultant products. In particular, this invention relates to blood plasma or other plasma protein-containing compositions which are to be rendered substantially free of hepatitis B and/or non-A and non-B hepatitis or other viral infectivity, such blood plasma or fractions thereof having valuable labile proteins, such as, for example, factor VIII.

2. Discussion of Prior Art

Numerous attempts have been made to inactivate viruses such as hepatitis B virus (HBV) in mammalian, especially human, blood plasma. It is the practice in some countries to effect inactivation of the hepatitis B virus in the blood plasma by contacting the plasma with a viral inactivating agent of the type which crosslinks with the proteinaceous portion of hepatitis B virus, or which interacts with the nucleic acid of the virus. For instance, it is to attempt to inactivate hepatitis B virus by contact an aldehyde such as formaldehyde whereby crosslinking to the protein is effected and the hepatitis B virus is inactivated. It is also known to effect inactivation of the virus by contact with beta-propiolactone (BPL), an agent which acts on the nucleic acid of the virus. It is further known to use ultraviolet (UV) light, especially after a beta-propiolactone treatment.

Unfortunately, these agents often alter, denature or destroy valuable protein components especially so-called "labile" blood coagulation factors of the plasma under conditions required for effective inactivation of virus infectivity. For instance, in such inactivation procedures, factor VIII is inactivated or denatured to the extent of 50–90% or more of the factor VIII present in the untreated plasma. Because of the denaturing effects of these virus inactivating agents, it is necessary in the preparation of derivatives for administration to patients to concentrate large quantities of plasma so that the material to be administered to the patient once again has a sufficient concentration of the undenatured protein for effective therapeutic treatment. This concentration, however, does not affect reduction of the amount of denatured protein. As a result, the patient not only receives the undenatured protein but a quantity of denatured protein often many times that of the undenatured protein.

For instance, in the inactivation of hepatitis B virus in human blood plasma by beta-propiolactone, there is obtained as a result thereof, a plasma whose factor VIII has been 75% inactivated. The remaining 25% of the factor VIII is therefore present in such a small concentration, as a function of the plasma itself, that it is necessary to concentrate large quantities of the factor VIII to provide sufficient concentration to be of therapeutic value. Since such separation techniques do not efficiently remove denatured factor VIII from undenatured factor VIII, the material administered to the patent may contain more denatured protein than undenatured protein. Obviously, such inactivation is valuable from a standpoint of diminishing the risk of hepatitis virus infection. However, it requires the processing of large quantities of plasma and represents significant loss of valuable protein components. Furthermore, administration of large amounts of denatured proteins may render these antigenic to the host and thus give rise to autoimmune diseases, or perhaps, rheumatoid arthritis.

The loss of these valuable protein components is not limited to factor VIII, one of the most labile of the valuable proteins in mammalian blood plasma. Similar protein denaturation is experienced in respect of the following other valuable plasma components: coagulation factors II, VII, IX, X; plasmin, fibrinogen (factor I) IgM, hemoglobin, interferon, etc.

Factor VIII, however, is denatured to a larger extent than many of the other valuable proteins present in blood plasma.

As a result of the foregoing, except in the processing of serum albumin, a stable plasma protein solution which can withstand pasteurization, it is largely the practice in the United States in respect of the processing of blood proteins to take no step in respect of the sterilization for inactivation of viruses. As a result, recipients of factor VIII, gamma-globulin, factor IX, fibrinogen, etc., must accept the risk that the valuable protein components being administered may be contaminated with hepatitis viruses as well as other infectious viruses. As a result, these recipients face the danger of becoming infected by these viruses and having to endure the damage which the virus causes to the liver and other organ systems and consequent incapacitation and illness which may lead to death.

The BPL/UV inactivation procedure discussed above has not so far been adopted in the United States for numerous reasons, one of which lies in the fact that many researchers believe that BPL is itself deleterious since it cannot be removed completely following the inactivation and thus may remain in plasma and plasma derivatives. BPL has been shown to be carcinogenic in animals and is dangerous even to personnel handling it.

Other methods for the inactivation of hepatitis B virus in the plasma are known, but are usually impractical. One method involves the addition of antibodies to the plasma whereby an immune complex is formed. The expense of antibody formation and purification add significantly to the cost of the plasma production; furthermore, there is no assurance that a sufficient quantity of hepatitis B or non-A, non-B virus is inactivated. There is currently no test for non-A, non-B antibodies (although there is a test for the virus); hence, it is not possible to select plasma containing high titers of anti non-A, non-B antibody.

It is to be understood that the problems of inactivation of the viruses in plasma are distinct from the problems of inactivation of the viruses themselves due to the copresence of the desirable proteinaceous components of the plasma. Thus, while it is known how to inactivate the hepatitis B virus, crosslinking agents, for example, glutaraldehyde, nucleic acid reacting chemicals, for example BPL or formaldehyde, or oxidizing agents, for example chlorox, etc., it has been believed that these methods are not suitable for the inactivation of the virus in plasma due to the observation that most of these

activating agents (sodium hypochlorite, formaldehyde, beta-propiolactone) denatured the valuable proteinaceous components of the plasma.

U.S. Pat. No. 4,315,919 to Shanbrom describes a method of depyrogenating a proteinaceous biological or pharmaceutical product by contacting such proteinaceous product with a non-denaturing amphiphile.

U.S. Pat. No. 4,314,997 to Shanbrom describes a method of reducing pyrogenicity, hepatitis infectivity and clotting activation of a plasma protein product by contacting the product with a non-denatured amphiphile.

Both Shanbrom '919 and '997 contemplate the use of a non-ionic detergent, for example, "Tween 80" as the amphiphile. It will be shown hereinafter that treatment with "Tween 80" by itself is relatively ineffective as a viral inactivating agent.

U.S. Pat. No. 3,962,421 describes a method for the disruption of infectious lipid-containing viruses for preparing sub-unit vaccines by contacting the virus in an aqueous medium with a wetting agent and a trialkylphosphate. Such aqueous medium is defined as allantonic fluid, tissue culture fluid, aqueous extract or suspension of central nervous system tissue, blood cell eluate and an aqueous extract or suspension of fowl embryo. The patent does not describe hepatitis, nor is it concerned with preparation of blood derivatives containing labile blood protein substantially free of viral infectivity. It is only concerned with disrupting the envelope of lipid containing viruses for the production of vaccines and not with avoiding or reducing protein denaturation en route to a blood derivative.

Problems may also exist in deriving valuable proteins from non-blood sources. These sources include, but are not limited to, mammalian milk, ascitic fluid, saliva, placental extracts, tissue culture cell lines and their extracts including transformed cells, and products of fermentation. For instance, the human lymphoblastoid cells have been isolated which produce alpha interferon. However, the cell line in commercial use today contains Epstein-Barr virus genes. It has been a major concern that the use of interferon produced by these cells would transmit viral infection or induce viral caused cancerous growth.

The present invention is directed to achieving three goals, namely, (1) a safe, (2) viral inactivated protein-containing composition, (3) without incurring substantial protein denaturation. As shown above these three goals are not necessarily compatible since, for example beta-propiolactone inactivates viral infectivity, but is unsafe and substances such as formaldehyde inactivate viruses, but also substantially denaturate the valuable plasma proteins, for example, factor VIII.

It, therefore, became desirable to provide a process for obtaining protein-containing compositions which does not substantially denature the valuable protein components therein and which does not entail the use of a proven carcinogenic agent. More especially, it is desirable to provide blood protein-containing compositions in which substantially all of the hepatitis viruses and other viruses present are inactivated and in which denatured protein such as factor VIII account for only a small amount of the total amount of these proteins in the blood protein-containing composition.

It is a further object to provide products from cancer or normal cells or from fermentation processes following gene insertion which are substantially free of virus, especially lipid-containing viruses.

SUMMARY OF THE INVENTION

It has now been discovered, quite surprisingly, that while most of the viral inactivating agents denature factor VIII and other valuable blood plasma proteins, that not all viral inactivating agents have such effect. It has been discovered that a protein-containing composition such as whole blood, blood cell proteins, blood plasma, a blood plasma fractionation precipitate, a blood plasma fractionation supernatant, cryoprecipitate, cryosupernatant, or portion or derivative thereof or serum or a non-blood product produced from normal or cancerous cells (e.g. via recombinant DNA technology) is contacted for a sufficient period of time with a dialkylphosphate or a trialkylphosphate that lipid containing viruses such as the hepatitis viruses present in the composition are virtually entirely inactivated without substantial denaturation of proteins therein. By contacting blood protein mixture or concentrate thereof or fraction thereof with a di-or trialkylphosphate, followed by removal of the di- or trialkylphosphate, hepatitis viruses can be substantially inactivated, e.g., to an inactivation of greater than 4 logs, while realizing a yield of protein activity to total protein of at least 80%.

By such procedures there is provided a blood protein-containing composition such as mammalian whole blood, blood cell derivatives (e.g., hemoglobin, alpha-interferon, T-cell growth factor, platelet-derived growth factor, etc.), plasminogen activator, blood plasma, blood plasma fraction, blood plasma precipitate (e.g., cryoprecipitate, ethanol precipitate or polyethylene glycol precipitate), or supernatant (e.g., cryosupernatant, ethanol supernatant or polyethylene glycol supernatant), characterized by the presence of one or more blood proteins such as labile blood factor VIII having a total yield of protein activity to total protein of at least 80%, preferably at least 85%, more preferably 95% and most preferably 98% to 100%, said blood protein-containing composition having greatly reduced or virtually no hepatitis viruses. Virus in a serum is determined by infectivity titrations.

By the inactivation procedure of the invention, most if not virtually all of the hepatitis viruses contained therein would be inactivated. The method for determining infectivity levels by in vivo chimpanzees is discussed by Prince, A. M., Stephen, W., Brotman, B. and van den Ende, M. C., "Evaluation of the Effect of Betapropiolactone/Ultraviolet Irradiation (BPL/UV) Treatment of Source Plasma on Hepatitis Transmission by factor IX Complex in Chimpanzees, Thrombosis and Haemostasis", 44: 138–142, 1980.

The hepatitis virus is inactivated by treatment with the di- or trialkylphosphate described herein, and is not inactivated because of inclusion in the plasma of antibodies which bind with the hepatitis viruses and form immune complexes.

Inactivation of virus is obtained to the extent of at least "4 logs", i.e., virus in a serum is totally inactivated to the extent determined by infectivity studies where that virus is present in the untreated serum in such a concentration that even after dilution to 10^4, viral activity can be measured.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows virus inactivation as a function of log titer value versus time for VSV virus (vesicular stomatitis virus) treated according to the present invention and treated with ether/Tween 80. The lower log titer for

treatment according to the present invention indicates greater virus inactivation;

FIG. 2 shows virus inactivation as a function of log titer value versus time for Sindbis virus treated according to the present invention and treated with ether/Tween 80;

FIG. 3 shows virus inactivation as a function of log titer value versus time for Sendai virus treated according to the present invention and treated with ether/Tween 80:

FIG. 4 shows virus inactivation as a function of log titer value versus time for EMC virus (a non-lipid coated virus) treated according to the present invention and treated with ether/Tween 80;

FIG. 5 is a plot of log titer value versus hours for VSV virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature);

FIG. 6 is a plot of log titer value versus hours for Sindbis virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature);

FIG. 7 is a plot of log titer value versus hours for Sendai virus for TNBP /Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature); and

FIG. 8 is a plot of log titer value versus hours for EMC virus for TNBP/Tween 80 at 0° C. and at room temperature and TNBP alone (at room temperature).

The Sindbis, Sendai and VSV viruses are typical lipid containing viruses and are used herein to determine the effect of di- or trialkylphosphate on lipid coated viruses generally.

DETAILED DESCRIPTION OF THE INVENTION

Blood is made up of solids (cells, i.e., erythrocytes, leucocytes, and thrombocytes) and liquid (plasma). The cells contain potentially valuable substances such as hemoglobin, and they can be induced to make other potentially valuable substances such as interferons, growth factors, and other biological response modifiers. The plasma is composed mainly of water, salts, lipids and proteins. The proteins are divided into groups called fibrinogens, serum globulins and serum albumins. Typical antibodies (immune globulins) found in human blood plasma include those directed against infectious hepatitis, influenza H, etc.

Blood transfusions are used to treat anemia resulting from disease or hemorrhage, shock resulting from loss of plasma proteins or loss of circulating volume, diseases where an adequate level of plasma protein is not maintained, for example, hemophilia, and to bestow passive immunization.

Whole blood must be carefully typed and cross matched prior to administration. Plasma, however, does not require prior testing. For certain applications, only a proper fraction of the plasma is required, such as factor VIII for treatment of hemophilia or von Willebrand's disease.

With certain diseases one or several of the components of blood may be lacking. Thus the administration of the proper fraction will suffice, and the other components will not be "wasted" on the patient; the other fractions can be used for another patient. The separation of blood into components and their subsequent fractionation allows the proteins to be concentrated, thus permitting concentrates to be treated. Of great importance, too, is the fact that the plasma fractions can be stored for much longer periods than whole blood and they can be distributed in the liquid, the frozen, or the dried state. Finally, it allows salvaging from blood banks the plasma portions of outdated whole blood that are unsafe for administration as whole blood.

Proteins found in human plasma include prealbumin, retinol-binding protein, albumin, alpha-globulins, beta-globulins, gamma-globulins (immune serum globulins), the coagulation proteins (antithrombin III, prothrombin, plasminogen, antiemophilic factor-factor VIII, fibrin-stabilizing factor-factor XIII, fibrinogen), immunoglobins(immunoglobulins G, A, M, D, and E), and the complement components. There are currently more than 100 plasma proteins that have been described. A comprehensive listing can be found in "The Plasma Proteins", ed. Putnam, F. W., Academic Press, New York (1975).

Proteins found in the blood cell fraction include hemoglobin, fibronectin, fibrinogen, enzymes of carbohydrate and protein metabolism, etc. In addition, the synthesis of other proteins can be induced, such as interferons and growth factors .

A comprehensive list of inducible leukocyte proteins can be found in Stanley Cohen, Edgar Pick, J. J. Oppenheim, "Biology of the Lymphokines", Academic Press, N.Y. (1979).

Blood plasma fractionation generally involves the use of organic solvents such as ethanol, ether and polyethylene glycol at low temperatures and at controlled pH values to effect precipitation of a particular fraction containing one or more plasma proteins. The resultant supernatant can itself then be precipitated and so on until the desired degree of fractionation is attained. More recently, separations are based on chromatographic processes. An excellent survey of blood fractionation appears in *Kirk-Othmer's Encyclopedia of Chemical Technology*, Third Edition, Interscience Publishers, Volume 4, pages 25 to 62, the entire contents of which are incorporated by reference herein.

The major components of a cold ethanol fractionation are as follows:

Fraction	Proteins
I	fibrinogen; cold insoluble globulin; factor VIII; properdin
II and III	IgG; IgM; IgA; fibrinogen; beta-lipoprotein; prothrombin; plasminogen; plasmin inhibitor; factor V; factor VII; factor IX; factor X; thrombin; antithrombin; isoagglutinins; ceruloplasmin; complement C'1, C'3
IV-1	$alpha_1$-lipoprotein, ceruloplasmin, plasmin-inhibitor; factor IX; peptidase; alpha-and-beta-globulins
IV-4	transferrin; thyroxine binding globulin; serum esterase; $alpha_1$-lipoprotein; albumin; alkaline phosphatase
V	albumin; alpha-globulin
VI	$alpha_1$-acid glycoprotein; albumin

The above fractionation scheme can serve as a basis for further fractionations. Fraction II and III, for example, can be further fractionated to obtain immune serum globulin (ISG).

Another fractionation scheme involves use of frozen plasma which is thawed into a cryoprecipitate containing AHF (antihemophilic factor) and fibronectin and a cryosupernatant. The cryoprecipitate is then fractionated into fibronectin and AHF.

Polyethylene glycol has been used to prepare high purity AHF and non-aggregated ISG.

High risk products with respect to the transmission of hepatitis B and non-A, non-B are fibrinogen, AHF and prothrombin complex, and all other blood protein preparations except immune serum globulin and, because they are pasteurized, albumin solutions. Hepatitis tests presently available can indicate the presence of hepatitis B surface antigen, but there is presently no screening test for non-A, non-B hepatitis.

The present invention is directed to contacting with di- or trialkylphosphate a blood protein-containing composition such as whole mammalian blood, blood cells thereof, blood cell proteins, blood plasma thereof, precipitate from any fractionation of such plasma, supernatant from any fractionation of such plasma, cryoprecipitate cryosupernatant or any portions or derivatives of the above that contain blood proteins such as, for example, prothrombin complex (factors II, VII IX and X) and cryoprecipitate (factors I and VIII). The present invention is also concerned with contacting di- or trialkylphosphate with a serum containing one or more blood proteins. Furthermore, the present invention is directed to contacting di- or trialkylphosphate with a blood protein-containing fraction containing at least one blood protein such as the following: factor II, factor VII, factor VIII, factor IX, factor X, fibrinogen and IgM. Additionally, the present invention concerns contacting a cell lysate or proteins induced in blood cells with di- or trialkylphosphate.

Such blood protein-containing composition is contacted with a dialkylphosphate or a trialkylphosphate having alkyl groups which contain 1 to 10 carbon atoms, especially 2 to 10 carbon atoms. Illustrative members of trialkylphosphates for use in the present invention include tri-(n-butyl) phosphate, tri-(t-butyl) phosphate, tri-(n-hexyl) phosphate, tri-(2-ethylhexyl) phosphate, tri-(n-decyl) phosphate, just to name a few. An especially preferred trialkylphosphate is tri-(n-butyl) phosphate. Mixtures of different trialkylphosphates can also be employed as well as phosphates having alkyl groups of different alkyl chains, for example, ethyl, di(n-butyl) phosphate. Similarly, the respective dialkylphosphates can be employed including those of different alkyl group mixtures of dialkylphosphate. Furthermore, mixtures of di- and trialkylphosphates can be employed.

Di- or trialkylphosphates for use in the present invention are employed in an amount between about 0.01 mg/ml and about 100 mg/ml, and preferably between about 0.1 mg/ml and about 10 mg/ml.

The di- or trialkylphosphate can be used with or without the addition of wetting agents. It is preferred, however, to use di- or trialkylphosphate in conjunction with a wetting agent. Such wetting agent can be added either before, simultaneously with or after the di- or trialkylphosphate contacts the blood protein-containing composition. The function of the wetting agent is to enhance the contact of the virus in the blood protein-containing composition with the di- or trialkylphosphate. The wetting agent alone does not adequately inactivate the virus.

Preferred wetting agents are non-toxic detergents. Contemplated nonionic detergents include those which disperse at the prevailing temperature at least 0.1% by weight of the fat in an aqueous solution containing the same when 1 gram detergent per 100 ml of solution is introduced therein. In particular there is contemplated detergents which include polyoxyethylene derivatives of fatty acids, partial esters of sorbitol anhydrides, for example, those products known commercially as "Tween 80", "Tween 20" and "polysorbate 80" and nonionic oil soluble water detergents such as that sold commercially under the trademark "Triton X 100" (oxyethylated alkylphenol). Also contemplated is sodium deoxycholate as well as the "Zwittergents" which are synthetic zwitterionic detergents known as "sulfobetaines" such as N-dodecyl-N, N-dimethyl-2-ammonio-1 ethane sulphonate and its congeners or nonionic detergents such as octyl-beta-D-glucopyranoside.

Substances which might enhance the effectiveness of alkylphosphates include reducing agents such as mercaptoethanol, dithiothreitol, dithioerythritol, and dithiooctanoic acid. Suitable nonionic surfactants are oxyethylated alkyl phenols, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene acids, polyoxyethylene alcohols, polyoxyethylene oils and polyoxyethylene oxypropylene fatty acids. Some specific examples are the following:

alkylphenoxypolyethoxy (30) ethanol
polyoxyethylene(2) sorbitan monolaurate
polyoxyethylene (20) sorbitan monopalmitate
polyoxyethylene (20) sorbitan monostearate
polyoxyethylene (20) sorbitan tristearate
polyoxyethylene (20) sorbitan monooleate
polyoxyethylene (20) sorbitan trioleate
polyoxyethylene (20) palmitate
polyoxyethylene (20) lauryl ether
polyoxyethylene (20)cetyl ether
polyoxyethylene (20) stearyl ether
polyoxyethylene (20) oleyl ether
polyoxyethylene (25) hydrogenated castor oil
polyoxyethylene (25) oxypropylene monostearate

The amount of wetting agent, if employed, is not crucial, for example, from about 0.001% to about 10%, preferably about 0.01 to 1.5%, can be used.

Di- and trialkylphosphates may be used in conjunction with other inactivating agents such as alcohol or ethers with or without the copresence of wetting agents in accordance with copending application Ser. No. 368,250 entitled "Sterilized Plasma and Plasma Derivatives and Process Therefor", assigned to the assignee hereof.

The ether or alcohol can be added in an amount of 1 to 50%, preferably 5 to 25% by weight, based on the volume of blood plasma, or concentrate or other blood plasma protein-containing composition to be treated.

Particularly contemplated ethers for inactivation use in accordance with the invention are those having the formula

$$R^1{-}O{-}R^2$$

wherein

R^1 and R^2 are independently C_1–C_{18} alkyl or alkenyl which can contain an O or S atom in the chain, preferably C_1–C_8 alkyl or alkenyl. Especially contemplated ethers are dimethyl ether, diethyl ether, ethyl propyl ether, methyl-butyl ether, methyl isopropyl ether and methyl isobutyl ether.

Alcohols contemplated include those of the formula

$$R^3OH$$

wherein

R^3 is a C_1 to C_{18} alkyl or alkenyl radical which can contain one or more oxygen or sulfur atoms in the chain

and which can be substituted by one or more hydroxyl groups.

Especially contemplated alcohols are those where the alkyl or alkenyl group is between 1 and 8 atoms. Particularly contemplated alcohols include methanol, ethanol, propanol, isopropanol, n-butanol, isobutanol, n-pentanol and the isopentanols. Also contemplated are compounds such as ethylene glycol, 1,2-propylene glycol, 1,3-propane diol, 1,4-butanediol, 2-hydroxy isobutanol (2-methy, 1,2-dihydroxypropane).

Treatment of blood protein-containing compositions with trialkylphosphate is effected at a temperature between −5° C. and 70°, preferably between 0° C. and 60° C. The time of such treatment (contact) is for at least 1 minute, preferably at least 1 hour and generally 4 to 24 hours. The treatment is normally effective at atmospheric pressure, although subatmospheric and superatmospheric pressures can also be employed.

Normally, after the treatment, the trialkylphosphate and other inactivating agents, for example, ether, are removed, although such is not necessary in all instances, depending upon the nature of the virus inactivating agents and the intended further processing of the blood plasma protein-containing composition.

To remove ether from plasma the plasma is generally subjected to a temperature of 4° C. to 37° C. with a slight vacuum imposed to draw off residual ether. Preferably means are provided to spread the plasma as a thin film to insure maximum contact and removal of the ether. Other methods for removal of ether in activating agents include:

(1) bubbling of nitrogen gas;
(2) diafiltration using ether insoluble, e.g. "TEFLON", microporous membranes which retain the plasma proteins;
(3) absorption of desired plasma components on chromatographic or affinity chromographic supports;
(4) precipitation, for example, by salting out of plasma proteins;
(5) lyophilization, etc.

When alcohol or nonionic detergents are employed with the trialkylphosphate they are removed by (2) to (5) above.

Di- or trialkylphosphate can be removed as follows:

(a) Removal from AHF can be effected by precipitation of AHF with 2.2 molal glycine and 2.0M sodium chloride
(b) Removal from fibronectin can be effected by binding the fibronectin on a column of insolubilized gelatin and washing the bound fibronectin free of reagent.

Generally speaking, any ether present is initially removed prior to removal of any detergent. The ether may be recovered for reuse by the use of suitable distillation/condenser systems well known to the art.

Alcohol is normally removed together with detergent. If the detergent includes both alcohol and ether, the ether is normally removed before the alcohol.

The process of the invention can be combined with still other modes of inactivating viruses including those for non-lipid coated viruses. For instance, a heating step can be effected in the presence of a protein stabilizer, e.g., an agent which stabilizes the labile protein (AHF) against inactivation by heat. Moreover, the heating can be carried out using stabilizers which also tend to protect all protein, including components of the virus, against heat if the heating is carried out for a sufficient length of time, e.g., at least 5 hours and preferably at least 10 hours at a temperature of 50°–70° C., especially 60° C. By such mode the virus is preferentially inactivated, nevertheless, while the protein retains a substantial amount, e.g., ≧80% of its protein activity. Of course, the best treatment can also be carried out simultaneously with the alkyl phosphate treatment.

The treatment of plasma or its concentrates, fractions or derivatives in accordance with the present invention can be effected using di- or trialkylphosphate immobilized on a solid substrate. The same can be fixed to a macro-molecular structure such as one of the type used as a backbone for ion exchange reactions, thereby permitting easy removal of the trialkylphosphate from the plasma or plasma concentrate. Alternatively the phosphate can be insolubilized and immobilized on a solid support such as glass beads, etc., using silane or siloxane coupling agents.

The method of the present invention permits the pooling of human blood plasma and the treatment of the pooled human blood plasma in the form of such pooled plasma. It also permits the realization of blood product derivatives such as factor VIII, gamma globulin, factor IX or the prothrombin complex (factors II, VII, IX, X), fibrinogen and any other blood derivative including HBsAg used for the preparation of HBV vaccine, all of which contain little or no residual infective hepatitis or other viruses.

The present invention is directed, inter alia, to producing a blood plasma protein-containing composition such as blood, blood plasma, blood plasma fractions, etc., which is substantially free of infectious virus, yet which contains a substantial amount of viable (undenatured) protein. More particularly, the present invention is directed to inactivation of lipid-containing virus and preferentially inactivation of hepatitis B and non-B, non-A virus. Other viruses inactivated by the present invention include, for example, cytomegaloviruses, Epstein Barr viruses, lactic dehydrogenase viruses, herpes group viruses, rhabdoviruses, leukoviruses, myxoviruses, alphaviruses, Arboviruses (group B), paramyxoviruses, arenaviruses, and coronaviruses.

According to the present invention, there is contemplated a protein-containing composition—a product produced from normal or cancerous cells or by normal or cancerous cells (e.g., via recombinant DNA technology), such as mammalian blood, blood plasma, blood plasma fractions, precipitates from blood fractionation and supernatants from blood fractionation having an extent of inactivation of virus greater than 4 logs of virus such as hepatitis B and non-A, non-B, and having a yield of protein activity to total protein of at least 80%, preferably at least 95% and most preferably 98% to 100%.

Further contemplated by the present invention is a composition containing factor VIII which is substantially free of hepatitis virus to the extent of having an inactivation of greater than 4 logs of the virus and a yield of protein activity to total protein of at least 80%, preferably at least 85%, more preferably at least 95% and most preferably 98% to 100%.

The process of the present invention has been described in terms of treatment of plasma, plasma fractions, plasma concentrates or components thereof. The process, however, is also useful in treating the solid components of blood, lysates or proteins secreted by cells. Thus, also contemplated are treatment of platelet concentrates, white cell (leukocyte) concentrates, and leukocyte-poor packed red cells as well as platelet rich

plasma, platelet concentrates and platelet poor plasma including packed cell masses comprising the white buffy coat consisting of white blood cells above packed red cells. Also contemplated is the treatment of masses containing concentrates of granulocytes, monocytes, interferon, and transfer factor.

One can treat plasma itself according to the present invention or fresh frozen plasma, thawed frozen plasma, cryoprecipitate, cryosupernatants or concentrates from frozen plasma as well as dilution products thereof.

By the same manipulative steps discussed above, virus present in products of normal or cancerous cells can be inactivated while retaining labile protein activity in such products. For instance, by the same di- or trialkylphosphate treatment one can inactivate products produced using normal or cancer cells, the exudate from normal or cancerous cells, hybridomas and products produced by gene splicing. Such treatment does not substantially adversely affect the desired protein. Cells used for production of desired protein can, of course, be mammalian as well as non-mammalian cells.

Factor VIII and factor IX coagulant activities are assayed by determining the degree of correction in APTT time of factor VIII—and factor IX—deficient plasma, respectively. J. G. Lenahan, Phillips and Phillips, *Clin. Chem.*, Vol. 12, page 269 (1966).

The activity of proteins which are enzymes is determined by measuring their enzymatic activity. Factor IX's activity can be measured by that technique.

Binding proteins can have their activities measured by determining their kinetics and affinity of binding to their natural substrates.

Lymphokine activity is measured biologically in cell systems, typically by assaying their biological activity in cell cultures.

Protein activity generally is determined by the known and standard modes for determining the activity of the protein or type of protein involved.

In order to more fully illustrate the nature of the invention and the manner of practicing the same, the following non-limiting examples are presented:

EXAMPLE 1

AHF solutions were incubated with 0.1% TNPB plus 1% Tween 80 for 18 hours at 4° C. These solutions were initially contacted with VSV virus, Sindbis virus and Sendai virus and thereafter brought in contact with an aqueous solution containing 0.1 weight percent of tri(n-butyl) phosphate (TNBP) and 1.0 weight percent detergent (Tween 80), with the following resultant virus inactivations: 4.7 logs of vesicular stomatitis virus (VSV), 5.8 logs of Sindbis virus, and 5.0 logs of Sendai virus. The virus was added just prior to the addition of the TNBP-Tween 80. The yield of AHF (labile protein/total protein) was found to be 86%.

Controls in which TNBP and Tween 80 were omitted showed little if any viral inactivation.

The results for Example 1 are shown below in Table I:

TABLE I

Temperature	Time (Hrs)	AHF U/mL	AHF % Yld	Log Titer Virus VSV	Log Titer Virus Sindbis	Log Titer Virus Sendai
4° C.	Start	10.4	(100)	4.7	5.8	5.0
	3	—	—	0.9	−0.4	2.2
	6	—	—	0.6	−0.5	1.5
	18	8.9	86	<−0.5	0.5	−0.5

In FIG. 1, FIG. 2, and FIG. 3, the results of Example 1 are plotted and compared to virus inactivation with ether (20%)/Tween 80 (1%). It is seen that for VSV (FIG. 1), Sindbis (FIG. 2) and Sendai (FIG. 3), inactivation was greater (lower log titer value) for treatment according to the present invention (with TNBP) than with ether/Tween 80 treatment.

In Table II, the effect of a "Tween 80" alone in the inactivation of viruses is shown. The data shows that little if any inactivation is due to "Tween 80".

TABLE II

EFFECT OF TWEEN 80 (1%) ALONE ON VIRUS INACTIVATION

Experiment	Temperature (°C.)	Duration (Hrs)	Inactivation (log#) VSV	Inactivation (log#) Sindbis	Inactivation (log#) Sendai	Inactivation (log#) EMC
1	0° C.	3	0.3	0.0	0.0	0.4
2	0° C.	18	ND*	−0.1	0.7	0.5
	22° C.	18	ND*	−0.1	−0.3	0.0

#log titer control minus log titer treated
*not done

EXAMPLE 2

Example 1 was repeated, but at 22° C. The results for Example 2 are summarized below in Table III:

TABLE III

Temperature	Time (Hrs)	AHF U/mL	AHF % Yld	Log Titer Virus VSV	Log Titer Virus Sindbis	Log Titer Virus Sendai
22° C.	Untreated	8.3	(100)	4.4	5.1	5.0
	3	8.2	99	<−0.4	<−0.5	-3.8

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be had to the appended claims, rather than to the foregoing specification, an indicating the scope of the invention.

We claim:

1. A process for rendering a blood product which comprises a labile blood protein substantially free of lipid-containing viruses without incurring substantial protein denaturation which comprises contacting said blood product with an effective amount of a di- or trialkylphosphate for a period of time sufficient to render said blood product substantially free of lipid-containing viruses without incurring substantial protein denaturation.

2. A process according to claim 1 wherein di- or trialkylphosphate has alkyl groups which contain 1 to 10 carbon atoms.

3. A process according to claim 2 wherein said trialkylphosphate has alkyl groups which contain 2 to 10 carbon atoms.

4. A process according to claim 2 wherein said trialkylphosphate is tri-n-butyl phosphate.

5. A process according to claim 1 wherein said contacting is conducted in the presence of a wetting agent.

6. A process according to claim 5 wherein said wetting agent is a non-ionic detergent.

7. A process according to claim 5 wherein said wetting agent is added to said blood product prior to contacting said blood product with said di- or trialkylphosphate.

8. A process according to claim 5 wherein said wetting agent is added simultaneously with said di- or trialkylphosphate to said blood product.

9. A process according to claim 5 wherein said wetting agent is added after said di- or trialkylphosphate contacts said blood product.

10. A process according to claim 6 wherein said detergent is a partial ester of sorbitol anhydrides.

11. A process according to claim 1 further comprising conducting said contacting in the presence of lipid coated virus inactivating agent selected from the group consisting of ethers and alcohols.

12. A process according to claim 5 further comprising conducting said contacting in the presence of an inactivating agent selected from the group consisting of ethers and alcohols.

13. A process according to claim 1 wherein said blood protein is selected from the group consisting of whole blood, blood plasma, red blood cells, leucocytes, platelet concentrates, a plasma concentrate, a precipitate from any fractionation of such plasma, a supernatant from any fractionation of said plasma, a serum, a cryoprecipitate, a cell lysate and proteins induced in blood cells.

14. A process according to claim 1 wherein said labile blood protein is selected from the group consisting of fibrinogen, cold insoluble globulin, properdin, IgG, IgM, IgA, betaliproprotein, plasmin-inhibitor, factor V, thrombin, antithrombin, isoagglutinins, cerutoplasmin, alpha$_1$-lipoprotein, peptidase, transferrin, thyroxine binding globulin, serum esterase, alkaline phosphates, alpha$_1$-acid glycoprotein, factor II, factor VII, factor VIII, factor IX, factor X, factor XIII, factor I, immunoglobulins, prealbumin, retinol-binding protein, albumin, alpha-globulins, beta-globulins, gamma-globulins, factor III, the complement components, fibronectin, antithrombin III, hemoglobin, interferon, T-cell growth factor and plasminogen activator.

15. A process according to claim 1 wherein following said contacting with said di- or trialkylphosphate, said di- or trialkylphosphate is removed.

16. A process according to claim 1 wherein said period of time is between about 1 minute and about 30 hours.

17. A process according to claim 1 wherein said contacting is conducted at a temperature of between about 0° C. and about 70° C.

18. A process according to claim 1 wherein said di- or trialkylphosphate is present in an amount between about 0.001% and about 1%.

19. A process according to claim 13 wherein said blood product comprises factor VIII.

20. A process according to claim 13 wherein said blood product comprises factor IX.

21. A process according to claim 1 wherein said blood product is additionally heated for at least 5 hours at 50° to 70° C.

22. A process according to claim 22 wherein the composition which is heated comprises a protein stabilizer which stabilizes a protein against denaturation by heat.

23. A process according to claim 1 wherein said labile blood protein is factor VIII.

24. A process according to claim 1 wherein said labile blood protein is factor VII.

25. A process according to claim 1 wherein said labile blood protein is factor IX.

26. A process according to claim 1 wherein said labile blood protein is factor X.

27. A process according to claim 1 wherein said labile blood protein is fibrinogen.

28. A process according to claim 1 wherein said labile blood protein is fibronectin.

29. A process according to claim 1 wherein said labile blood protein comprises an antibody against infectious hepatitis.

30. A process according to claim 1 wherein said labile blood protein is factor XIII.

31. A process according to claim 1 wherein said labile blood protein is T-cell growth factor.

32. A process according to claim 1 wherein said labile blood protein is a protein of a leucocyte.

33. A process according to claim 1 wherein said labile blood protein is a protein of a red blood cell.

34. A process according to claim 1 wherein said labile blood protein is a protein of a blood platelet concentrate.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,540,573

DATED : September 10, 1985

INVENTOR(S) : Alexander R. Neurath, et al

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

FIG. 6 and FIG. 7	Delete "TNBP/TWEEN 80-°C" and substitute --TNBP/TWEEN 80-0°C--
Sheet 2 of Drawings	Bottom of page delete "TNBP/TWEEN 80-°C" and substitute --TNBP/TWEEN 80-0°C--

Signed and Sealed this

Eleventh Day of April, 1989

Attest:

DONALD J. QUIGG

Attesting Officer

Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,540,573

DATED : September 10, 1985

INVENTOR(S) : Alexander R. Neurath, et al.

It is certified that error appears in the above–identified patent and that said Letters Patent are hereby corrected as shown below:

Col. 14, line 14 Delete "claim 22" and substitute --claim 21--

Signed and Sealed this

Fifteenth Day of September, 1987

Attest:

DONALD J. QUIGG

Attesting Officer *Commissioner of Patents and Trademarks*

[redacted]

United States Patent [19]

Mitra et al.

[11] Patent Number: 4,762,714

[45] Date of Patent: Aug. 9, 1988

[54] PREPARATION OF RETROVIRUS-FREE IMMUNOGLOBULINS

[75] Inventors: Gautam Mitra, Kensington; Milton M. Mozen, Berkeley, both of Calif.

[73] Assignee: Miles Laboratories, Inc., Elkhart, Ind.

[21] Appl. No.: 849,612

[22] Filed: Apr. 8, 1986

[51] Int. Cl.[4] A61K 35/14; A61K 39/395; C07K 15/06; C12N 9/00

[52] U.S. Cl. 424/101; 424/85; 424/89; 435/236; 530/387; 514/2

[58] Field of Search 424/101, 85, 89; 435/236; 530/387; 514/2

[56] References Cited

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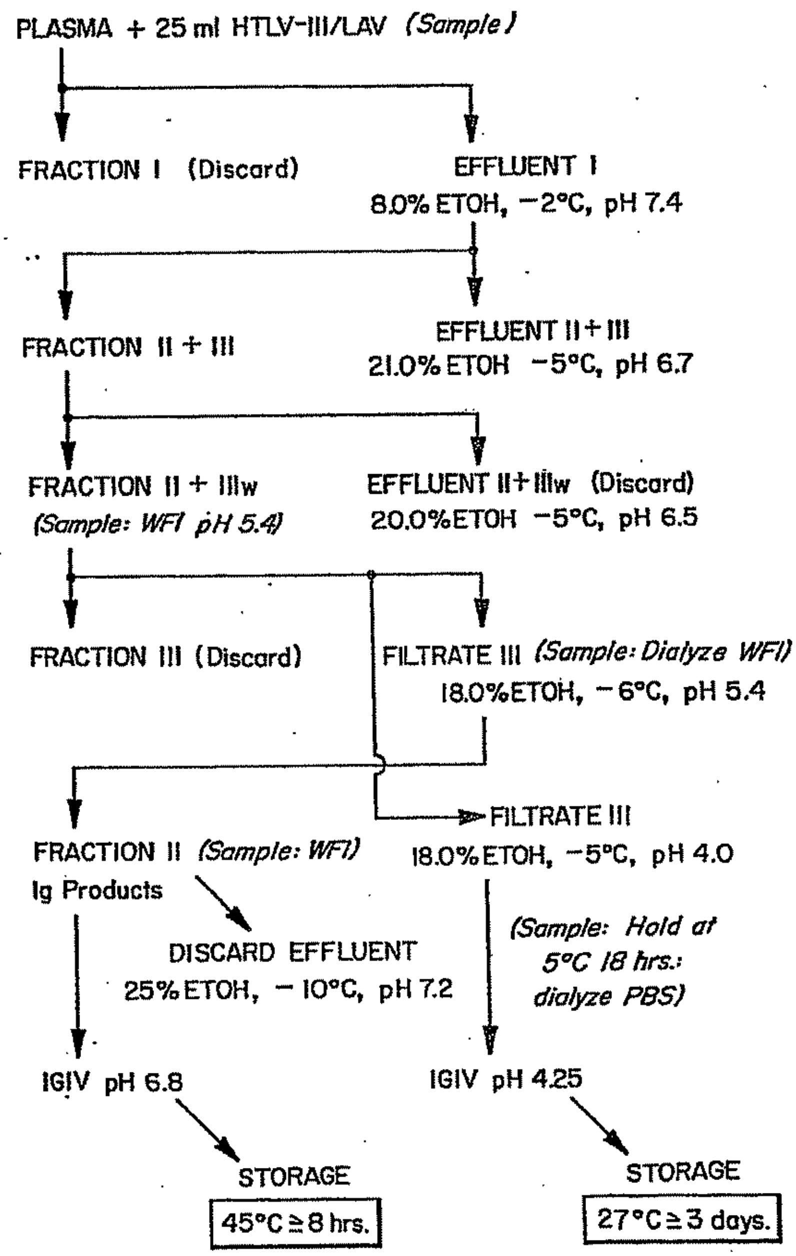
Primary Examiner—Alan Siegel
Assistant Examiner—Jacqueline M. Stone
Attorney, Agent, or Firm—James A. Giblin

[57] ABSTRACT

Immune serum globulins (ISG) can be made substantially free of infectious retroviruses by preparing the ISG from human plasma using a cold ethanol plasma fractionation process at a pH equal to or less than 5.4 and then storing the ISG at either of two specified storage conditions: (1) at a pH equal to or less than about 4.25 at a temperature of about 27° C. for at least 3 days, or (2) at a pH equal to or less than about 6.8 at a temperature of about 45° C. for at least about 8 hours.

2 Claims, 1 Drawing Sheet

HTLV—III/LAV Plasma Fractionation



PREPARATION OF RETROVIRUS-FREE IMMUNOGLOBULINSpg,10

BACKGROUND OF THE INVENTION

1. Field

This disclosure is concerned generally with the inactivation of retroviruses in immune serum globulin (ISG) and specifically with the inactivation of such retroviruses as the LAV strain of an AIDS virus in ISG intended for intravenous (IV) administration.

2. Prior Art

Therapeutic and prophylactic ISG preparations are well known and have been available for many years. ISG is presently obtained in commercial quantities using variations of a blood plasma fractionation technique developed by Cohn et al in the 1940's. Although ISG has been administered intramuscularly (IM) and more recently intravenously (IV), the latter route of administration provides numerous advantages and has gained acceptance as the preferred route of administration.

Initial attempts to render an ISG safe and effective for IV administration (IVIG) focused on eliminating its anticomplement activity. In one approach, for example, this involved chemically modifying the ISG (see U.S. Pat. No. 3,903,262 to Pappenhagen et al). More recently, the ISG has been made suitable for IV administration through careful pH and ionic strength control (see U.S. Pat. No. 4,396,608 and U.S. Pat. No. 4,499,073 both to Tenold). It is also known that IVIG preparations can be stabilized with carbohydrates such as maltose (see U.S. Pat. No. 4,186,192 to Fernandes et al). ISG preparations can be further purified using a variety of techniques (see, for example, U.S. Pat. No. 4,272,521 to Zuffi). Various ISG preparations having a relatively high titer to a given antigen are also well known (e.g. tetanus, hepatitis, Rho factor, etc.).

Although ISG products (both IMIG and IVIG) have been considered generally safe, there has been a growing need to assure patients that ISG products do not transmit active viruses such as those associated with hepatitis or, more recently, retroviruses such as that associated with Acquired Immune Deficiency Syndrome (AIDS). The present disclosure is based on work done to address such needs.

Antibodies to a retrovirus associated with the AIDS have been detected in human hepatitis B immunoglobulin (HBIG) (see Tedder, R. S. et al, Safety of immunoglobulin preparation containing anti-HTLV-III, Lancet 1985;1:815) as well as in other commercial lots of immunoglobulins (see Gocke, D. J. et al, HTLV-III antibody in commercial immunoglobulin, Lancet 1986;1:37-8). This observation raised the possibility that immunoglobulin products transmit infectious virus. This concern was heightened by recent reports of non A, non B (NANB) hepatitis in immunodeficient patients who had received infusions of intravenous immunoglobulins prepared from Cohn fraction II (see Webster, A. D. B. et al, Non-A, non-B hepatitis after intravenous gammaglobulin, Lancet 1986;i:322, and Ochs, H. D. et al, Non-A, non-B hepatitis after intravenous gammaglobulin, Lancet 1986;1:322-23).

Based on the above findings, we decided to determine the ability of retroviruses to withstand the various procedures employed in immunoglobulin preparations as well as other procedures. For these experiments, two prototype retroviruses were used: the mouse xenotropic type C retrovirus and the LAV strain of the AIDS retrovirus. Surprisingly, we found that the model retroviruses could be inactivated in ISG prepared by a known fractionation processing technique if that technique is followed by storage at controlled conditions of pH, temperature and time. Details of our method are described below.

SUMMARY OF THE INVENTION

We have found that ISG preparations can be made substantially free of retrovirus such as a LAV strain associated with AIDS by preparing the ISG from pooled plasma using a known processing technique (i.e. Cohn-Oncley cold ethanol process, using at least about 18% ethanol v/v at pH 5.4), followed by storage of the ISG at a pH of less than 5.4, a temperature of at least about 27° C., or at a pH of 6.8 at a temperature of at least about 45° C. for periods sufficient to assure retrovirus inactivation. In preferred embodiments, our ISG preparation is stabilized with a carbohydrate (e.g. maltose) and in a 5% wt./vol. liquid (aqueous) form. It is intended for IV use and is made substantially free (less than 10 infectious virus particles) of the LAV strain of retrovirus associated with AIDS by processing pooled human plasma using the Cohn-Oncley cold ethanol process (about 18% ethanol, pH≧5.4) to obtain ISG followed by storage of the ISG at a pH of about 4.25 for at least about 21 days at a temperature about 27° C. In another embodiment, the ISG may be stored at pH 6.8 for about 45° C., for at least 8 hours to assure the retrovirus inactivation.

BRIEF DESCRIPTION OF THE FIGURE

The FIGURE illustrates a flow chart of the steps used in our Cohn-Oncley cold ethanol fractionation of human plasma, including the novel storage conditions disclosed herein.

SPECIFIC EMBODIMENTS

Materials and Methods

The mouse xenotropic type C retrovirus recovered from a New Zealand Black mouse kidney was grown to high titer in mink lung cells (Varnier, O. E. et al, Murine xenotropic type C viruses. V. Biological and structural differences among three cloned retroviruses isolated from kidney cells from one NZB mouse, Virology 1984;132:79-94). Detection was based on a focus assay in mink S+L-cells in which each infectious particle scores as an area of cell transformation (Peeples, P. T., An in vitro focus induction assay for xenotropic murine leukemia virus, feline leukemia virus C, and the feline Primate viruses RI-114/CCC/M-7, Virology 1975;67:288-91). Virus titer was also determined by the induction in cells of the viral core structural protein (page 30) measured by immunofluorescence (see Levy, J. A., Xenotropic type C viruses, Current Topics Microbiol. Immunol. 1978;79:111-212). The use of these assays for detection of mouse C virus in spiking experiments with plasma fractions has previously been described by us (see Levy, J. A. et al, Recovery and inactivation of infectious retroviruses added to factor VIII concentrates, Lancet 1984;ii:722-723 and Levy, J. A. et al, Inactivation by wet and dry heat of AIDS-associated retroviruses during factor VIII purification from plasma, Lancet 1985;i:1456-1457).

LAV was cultured and obtained from the Centers for Disease Control (CDC) in Atlanta, Ga. Its detection

was based on a sandwich enzyme-linked immunoassay (ELISA) previously described (see McDougal, J. S. et al, Immunoassay for the detection and quantitation of infectious human retrovirus, lymphadenopathy-associated virus [LAV], J. Immunol. Methods 1985;76:171–183).

Human plasma samples were spiked with retroviral preparations and fractionated according to classical Cohn-Oncley cold ethanol procedures (see Cohn, E. J. et al, Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of protein and lipoprotein components of biological tissues and fluids, J. Am. Chem. Soc. 1946;68:459–75 and Oncley, J. L. et al, The separation of the antibodies, isoagglutinins, prothrombin, plasminogen, and beta-I-lipoprotein into subfractions of human plasma, J. Am. Chem. Soc. 1949;71-541-50). The fractionation was accomplished through selective precipitations in the cold at various ethanol concentrations and pH values: fraction I at 8% ethanol, −2° C., pH 7.4; fraction II+III at 21% ethanol, −5° C., pH 6.7; fraction II+IIIw at 20% ethanol, −5° C., pH 6.5; fraction III at 18% ethanol, −6° C., pH 5.4; and fraction II collected at 25% ethanol −10° C., pH 7.2. Residual retroviral levels were determined across the fractionation steps. The pH (range 5.4–4.0) and temperature (range −5° C. to 22° C.) effects on virus infectivity in the presence of ethanol (approximately 18%) were determined with filtrate III. Final container liquid immunoglobulin preparations, in the absence of ethanol, were incubated with retrovirus concentrates at 27° C. and 45° C.; virus infectivity was determined at different time periods.

Results

Infectivity of both the mouse C and AIDS retrovirus was not affected by the addition of these viruses to human plasma at ≦5° C. See Table 1.

TABLE 1

Effect of Immunoglobulin fractionation procedures on infectious retrovirus added to plasma

Store	Mouse Type C (Total IP)	AIDS Virus LAV (Total ID_{50})
Virus alone	2.0×10^8	2.3×10^5
Virus + plasma (5° C.)	2.3×10^8	4.4×10^5
II + IIIw	3.8×10^7	4.8×10^4
Filtrate III	1.6×10^3	1.7×10^3
Fraction II	Non-detectable	Non-detectable

Twenty ml of virus concentrate was added to 200 ml of plasma for the fractionation studies described. The fractionation methods and viral assays are described in the text. Total IP = total infectious particles. Total ID_{50} = ID_{50} (reciprocal of dilution at which 50% of the cultures are positive) × volume.

From plasma to fraction II+IIIw, no more than a 10-fold reduction of virus titer was observed. Preparation of filtrate III from fraction II+IIIw resulted in an approximately 10,000-fold reduction of the mouse type C retrovirus and 10-fold reduction in LAV. Due to dilution, ethanol concentration decreased from 20% v/v to 18% v/v across this fractionation step and the pH was reduced from 6.50 to 5.40. Fraction II precipitation from filtrate III resulted in >1,000-fold reduction in titer of both the infectious mouse and human retroviruses. During this fractionation step, the pH was raised to 7.25 and the ethanol concentration increased to 25%. The 1,000-fold loss of virus infectivity primarily results from virus inactivation (not fractionation) since after extensive dialysis, no infectious virus was measurable in the supernatant corresponding to fraction II (data not shown).

In studying more precisely the effect of pH and temperature on retrovirus inactivation with 18% ethanol, we mixed a quantity of the mouse retrovirus with filtrate III. See Table 2.

TABLE 2

Effect of pH and Temperature on Mouse Type C Retrovirus added to Filtrate III (18% Ethanol)

	Temperature −5° C.				Temperature 22° C.	
Sample	(a) pH 5.4 (Total IP)	(b) pH 4.7 (Total IP)	(c) pH 4.0 (Total IP)	Sample	(d) pH 5.4 (Total IP)	(e) pH 4.0 (Total IP)
Virus alone	7.9×10^6	7.9×10^6	7.9×10^6	Virus alone	4.0×10^8	5.0×10^7
Virus + filtrate III	2.9×10^6	3.4×10^5	6.5×10^5	Virus + filtrate III	2.2×10^9	5.5×10^5
2 hours	3.4×10^5	6.6×10^6	2.0×10^5	3 hours	2.2×10^8	Non-detectable
4 hours	6.7×10^5	6.7×10^5	2.6×10^5			
6 hours	7.8×10^5	6.6×10^5	4.1×10^5			

Total infectious particles were detected as described under Materials and Methods. Detection limit approximately $10^{-0.3}$ IP/m. Two ml of mouse type C virus concentrate was added to 20 ml of effluent III for each of (a), (b), and (c). Ten ml and 5 ml of mouse type C virus were added to 100 ml and 50 ml of effluent III, respectively for (d) and (e).

At −5° C., no significant virucidal effect was seen in the pH range of 5.4–4.0 for up to 6 hours (2*a*, *b*, *c*). At 22° C. (ambient), however, at pH 4.0>100,000 infectious mouse retrovirus particles were inactivated by 3 hours (2*e*). In contrast, at pH 5.4 under similar conditions, no significant virucidal effect was seen (2*d*). Similarly, 1.7×10^3 total ID_{50} of LAV that was in a filtrate III solution at pH 4.0 and held at +5° C. for 18 hours, was reduced in titer to non-detectable level (data not shown). It therefore appears that the presence of 18% ethanol in plasma fractions at pH 5.4 is not markedly virucidal for these viruses in the temperature range of −5° C. to 22° C. Only when the pH is lowered (pH 4.0) concomitant with a raise in temperature (≧5° C.), significant virus inactivation observed. For LAV, the following conditions were sufficient for a 1,000-fold reduction in infectious virus: ethanol 18%, pH 4.0, temperature +5° C., time 18 hours (data not shown). For the mouse type C retrovirus, >10,000-fold reduction was measured under similar treatment conditions. To determine the effect on AIDS virus of pH and temperature of the final product, final container liquid immunoglobulin preparations (protein concentration 5% w/v) were incubated with LAV (Table 3). At 27° C., between 10^3–10^4 of total ID_{50} were inactivated by 3 days for the immunoglobulin preparations of both pH 6.8 and pH 4.25. At 45° C., >10,000 infectious particles were inactivated within 8 hours with the pH 6.8 immunoglobulin preparation. The pH 4.25 immunoglobulin preparation was not tested at 45° C.

Discussion

These experiments were conducted to evaluate the effect on infectious retroviruses of procedures used for immunoglobulin fractionation. The data are important in evaluating the possible risk of AIDS virus contamination of some Ig preparations. The mouse type C retrovirus was used as well as the LAV strain of AIDS virus, because the former can be grown to very high titer and therefore the effect of various procedures can be better evaluated. In addition, a focus assay for the mouse virus allows more precise quantitation.

Unlike the reported complement-mediated lysis of many retroviruses in human serum at 37° C. (see Welsh, R. M. et al, Human serum lysis RNA tumor viruses, Nature 1975;257:612–14), the AIDS virus in the cold (0°–5° C.) is not affected by this mechanism (see Banapour, B. et al, The AIDS-associated retrovirus is not sensitive to lysis or inactivation by human serum, Virology [in press] 1986). The reported virucidal effects of ethanol for LAV have been at ambient temperature (see Spire, B. et al, Inactivation of lymphadenopathy associated virus by chemical disinfectants, Lancet 1984;ii:899–901 and Martin, L. S. et al, Disinfection and inactivation of human T lymphotropic virus type III/ lymphadenopathy associated virus, J. Infec. Dis. 1985;152:400403), whereas the data reported here show that these virus inactivating effects are diminished in the presence of plasma at low temperatures (<45° C.). Enhanced inactivation at low pH is demonstrated which again is strongly dependent on temperature. This observation agrees with an earlier report (see Martin, L. S. et al, Disinfection and inactivation of human T lymphotropic virus type III/lymphadenopathy associated virus, J. Infec. Dis. 1985; 152:400–403) indicating increased inactivation of LAV inoculum at pH extremes.

Filtrate III with 18% ethanol at pH 5.4 and at a temperature of −5° C. was not significantly virucidal for retroviruses for extended periods of time. Hence, the 100,000-fold reduction of the mouse type C virus and a 100-fold reduction of LAV from plasma to filtrate III is probably primarily due to fractionation under the processing condition (ethanol range 0–20% v/v, pH range 7.4–5.4) employed at −5° C. The reduction difference between the mouse and the human virus reflects either a greater resistance of the AIDS virus to the processing conditions or a less quantitative assay for this virus. As noted above, the mouse virus can be grown up to high titers and its assay is very reproducible. Its usefulness for fractionation/inactivation studies has been previously reported by us (see Levy, J. A. et al, Recovery and inactivation of infectious retroviruses added to factor VIII concentrates, Lancet 1984;ii:722–723 and Levy, J. A. et al, Inactivation by wet and dry heat of AIDS-associated retroviruses during factor VIII purification from plasma, Lancet 1985;i:1456–1457).

Ethanol concentration is increased to 25% v/v at pH 7.20 for the fraction II precipitation which results in a more than 1,000-fold inactivation of the mouse type C virus and LAV. Since the corresponding effluent was free of infectious virus, true inactivation at the 25% ethanol concentration is most likely involved. A recent report (see Piszkiewicz, D. et al, Inactivation of HTLV-III/LAV during Plasma fractionation, Lancet 1985;ii:1188–89) had shown inactivation of $10^{4.5}$ ID_{50} of the AIDS retrovirus during the precipitation of I+II+III (ethanol 20% v/v, pH 6.9, temperature −5° C.) under conditions in which fraction II+III is precipitated together with fraction I. Our results which isolate these fractions separately do not show such complete LAV inactivation under similar conditions (Table 1). In our study, the samples were extensively dialyzed in PBS prior to ID_{50} assay. In the other report, a 1:10 dilution to a resultant residual ethanol concentration of 2% v/v was used in the assay. Furthermore, it is not possible from the other report to distinguish whether the virus titer was being determined in the precipitate or the supernatant following I+II+III precipitation; hence, a meaningful comparison between the two studies is difficult to make.

Greater than a 1,000-fold drop in AIDS virus infectivity did result after its incubation with purified liquid immunoglobulin preparations at 27° C. for 3 days; pH of the purified immunoglobulin preparations did not seem to have an appreciable effect. A higher incubation temperature (45° C.) demonstrated comparable titer reduction within 8 hours. A "worse case" estimate of 2,000 ID/ml of AIDS virus in large plasma pools has been reported (see Petricciani, J. C. et al, Case for concluding that heat-treated, licensed antihaemophilic factor is free from HTLV-III, Lancet 1985;ii:890–891). The yield of IgG could be as low as 50% of the amount present in plasma together with IgG concentration increase from approximately 1 gm/100 ml in plasma to 5 gm/100 ml in purified product. If the AIDS virus was concentrated without loss of infectivity along with IgG purification, the purified IgG would contain 2,000 ID/ml×10 (2×10^4 ID/ml) Immunoglobulin purification processes must therefore be able to fractionate/inactivate 2×10^4 ID/ml of AIDS virus.

No single step in the Cohn cold ethanol process can completely inactivate retroviruses. The effects of fractionation and inactivation taken together through the fractionation cascade could be quite large. LAV recovery from plasma to fraction II is reduced by at least 100,000-fold; pH adjustment to 4.0 at filtrate III (at +5° C.) is as effective for viral inactivation as precipitation of fraction II in the presence of 25% ethanol. An extra margin of safety is provided when the final preparation in liquid form is incubated at 27° C., since these experiments demonstrated that in liquid immunoglobulin preparations, a 1,000–10,000-fold reduction of LAV occurred within 3 days under these conditions. Prince et al, Effect of Cohn fractionation conditions on infectivity of the AIDS virus. N. Eng. J. Med 1986; 314:386–87, have suggested that the long storage of liquid immune serum globulin preparations may contribute to their safety. The studies presented here experimentally validate that AIDS virus are indeed inactivated during liquid storage. See Table 3.

TABLE 3

Effect of pH and Temperature on LAV added to Final Container Liquid Immunoglobulin Preparations

	Temperature 27° C.			Temperature 45° C.
Sample	pH 6.8 IgG (Total ID_{50})	pH 4.25 IgG (Total ID_{50})	Sample	pH 6.8 IgG (Total ID_{50})
Virus + IgG	1.65×10^4	3.69×10^3	Virus + IgG	1.65×10^4
3 days	Non-detectable	Non-detectable	1 hour	6.27×10^3
12 days	Non-detectable	Non-detectable	4 hours	1.65×10^3
24 days	Non-detectable	Non-detectable	8 hours	Non-detectable
			20 hours	Non-

TABLE 3-continued

Effect of pH and Temperature on LAV added to Final Container Liquid Immunoglobulin Preparations

	Temperature 27° C.			Temperature 45° C.
Sample	pH 6.8 IgG (Total ID_{50})	pH 4.25 IgG (Total ID_{50})	Sample	pH 6.8 IgG (Total ID_{50})
				detectable

ID_{50} of LAV as defined in Table 1. ID_{50} detection limit $10^{1.0}$. 1.5 ml of an LAV preparation was added to 15 ml of IgG solution for each of the two parts of the experiment.

The chance for an infectious retrovirus to survive this fractionation as well as storage of the liquid final preparation, is therefore extremely small, if at all.

The fractionation/inactivation and final container incubation results reported here support the available clinical and epidemiological evidence that therapeutic immunoglobulins prepared by Cohn-Oncley cold ethanol process (≧18% v/v ethanol, pH≦5.4 at filtrate III) do not transmit AIDS viruses particularly after storage at a pH of 4.25 at a temperature of 27° C. for about 3 days or at pH 6.8 at temperature of 45° C. for at least 8 hours. The conditions of the Cohn-Oncley process i.e., alcohol concentration, pH, temperature, do not in themselves inactivate AIDS virus as recently reported by Prince et al, Effect of Cohn fractionation conditions on infectivity of the AIDS virus, N. Eng. J. Med. 1986;314:386–87. As described, their study was primarily geared towards determining inactivation, and no sequential fractionation was carried out with a virus spike. The present study, in contrast, mimics a true fractionation run and hence portrays a realistic virus

carryover estimate involving the sum total of fractionation and inactivation.

It is important to emphasize that variations from classical Cohn approach need to be validated in terms of their virucidal and virus distribution potential since fractionation, ethanol concentration, pH, and temperature all play an important role in virus recovery. It is possible that total log reduction of different viruses could be different and hence it would be difficult to generalize these virus recovery results for other viruses.

However, given the above disclosure, it is thought that variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention disclosed should be limited only by the following claims.

We claim:

1. A method of preparing an immune serum globulin substantially free of infectious retroviruses comprising the steps of

(1) preparing an immune serum globulin from a human plasma source using a cold ethanol process at a pH equal to or less than about 5.4 and then

(2) storing the globulin at a pH equal to or less than about 4.25 at a temperature of about 27° C. for at least about 3 days.

2. A method of preparing an immune serum globulin substantially free of infectious retroviruses comprising the steps of

(1) preparing an immune serum globulin from a human plasma source using a cold ethanol process at a pH equal to or less than about 5.4 and then

(2) storing the globulin at a pH equal to or less than about 6.8 at a temperature of about 45° C. for at least about 8 hours.

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Original Paper

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Antibody Fc Functional Activity of Intravenous Immunoglobulin Preparations Treated with Solvent-Detergent for Virus Inactivation

Abstract

We report here results of in vitro comparisons of the Fc functional activity of a second-generation intravenous immunoglobulin (IGIV) preparation (Venoglobulin®-I) and a third-generation IGIV product that includes a deliberate virus-inactivation step (Venoglobulin®-S). Both formulations showed equivalent Fc-mediated function against viral antigens (rubella, influenza A, and influenza B) by single-radial hemolysis test, and against group B *Streptococcus*, *Staphylococcus aureus* and *Escherichia coli* by opsonophagocytosis assay. In addition, we showed by three different immunochemical reactions and by HPLC analysis that both preparations consisted of mostly monomeric IgG and contained very low levels of complement-fixing IgG aggregates. However, IgG aggregation induced by heating at 63°C markedly enhanced fixation of C1q and C3 and binding to Raji cells, indicating that the IgG molecules retained their complement-fixing capacity. Thus, the incorporation of a virus inactivation step in the manufacture of our third-generation IGIV did not alter the Fc functional activities of the IgG, as measured by these in vitro assay systems.

Introduction

Intravenous immunoglobulin (IGIV) has been used to treat a wide variety of immunodeficiencies and other immunopathologic conditions [1–4]. When IGIV is used as antibody replacement therapy in primary or acquired immunodeficiency states, antibody is believed to provide protection by killing bacterial pathogens through effector systems such as complement-dependent cytotoxicity or opsonophagocytosis, which depend on the function of both the antigen-binding and Fc portion of the antibody molecule. In other conditions, such as idiopathic thrombocytopenic purpura and Kawasaki syndrome, IGIV is believed to act as an immunomodulating agent, through Fc receptor blockade [1–4]. Thus, it is clear the retention of the full range of Fc function is a prime requirement for therapeutic applications of IGIV.

During the 1980s, transmission of the human immunodeficiency virus (HIV) by blood and blood products [5–8] highlighted the need for viral inactivation procedures that could be incorporated into manufacturing procedures. Moreover, although IGIV products are generally considered to be safe products, transmission of hepatitis B and non-A, non-B (hepatitic C) viruses via such products has been reported [9–16]. Although the Cohn-Oncley fractionation procedures used to produce IGIV in the United States are apparently effective in inactivating HIV [17–19], hepatitis C virus (HCV) RNA has been detected in the immunoglobulin

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after alcohol fractionation [20]. Moreover, HCV RNA could be detected even in plasma pools taken from individuals screened for anti-HCV antibodies, suggesting that 'virus-inactivation and/or removal steps should be considered for immune globulin products' [20].

Investigators at the New York Blood Center have developed methods to inactivate lipid-enveloped human pathogenic virus in blood derivatives. These methods, based on solvent-detergent disruption of the virus lipid envelope, have been found to be effective in the inactivation of a wide variety of human pathogenic and experimental marker viruses, while retaining biological properties of a wide range of plasma derivatives, such as coagulation factors VII, VIII, IX, XIII, fibrinogen, fibronectin, immunoglobulin, haptoglobin, tumor necrosis factor, interferon-α, and hemoglobin [21–25]. Recently, we developed a process that incorporates solvent-detergent treatment into the manufacture of IGIV. We report here the results of studies designed to evaluate Fc function in IGIV preparations obtained by these methods.

Materials and Methods

Immunoglobulin Preparations

Venoglobulin®-I, Immune Globulin Intravenous (Human) (Alpha Therapeutic Corporation, Los Angeles, Calif., USA), is an intact, biologically active preparation of human immunoglobulin for intravenous use. Venoglobulin-I is prepared from pooled human plasma using the Cohn-Oncley cold ethanol fractionation process [26, 27]. Fraction II precipitate is further purified and immune complexes are removed by polyethylene glycol fractionation and ion exchange chromatography. Venoglobulin-I is supplied as a lyophylized powder. For the studies reported here, a 5% (weight to volume) solution of Venoglobulin-I was prepared according to the manufacturer's directions, and diluted as indicated for specific functional tests.

Venoglobulin®-S 5% Solution, Immune Globulin Intravenous (Human), Solvent Detergent Treated, is the first 'third-generation' IGIV product – fully intact, with an additional virus inactivation step. The production process for Venoglobulin-S is similar to that for Venoglobulin-I; however, the Venoglobulin-S process incorporates a solvent-detergent (tri-n-butyl phosphate plus polysorbate 80) treatment step to inactivate viruses, and an additional ion exchange chromatography step to eliminate solvent and detergent. Venoglobulin-S is supplied as a 5% (weight to volume) solution; this formulation was diluted as indicated for the functional assays described below.

At the time these studies were performed, three lots of IGIV paste for the preparation of Venoglobulin-I were available. The test samples were clinical lots used for the licensing studies of Venoglobulin-S (GL0005A, GL0006A, and GL0008) and production lots of Venoglobulin-I (GV0007A, GV0015A, and GV0017A). The following pairs of Venoglobulin-I and Venoglobulin-S were derived from the same lot of IGIV paste: GV0007A and GL0005A; GV0015A and GL0006A; and GV0017A and GL0008A. One lot of cryoprecipitate-poor human plasma pool (lot C-10183-B) was used as a reference.

Single Radial Hemolysis Test

The single radial hemolysis test was used to evaluate functional activity of IGIV preparations against rubella, influenza A, and influenza B viral antigens. The test was performed using modifications of previously published methods [28–30]. Briefly, undiluted or two-fold, serially diluted test samples were added to the wells of an agarose plate containing viral antigen-sensitized sheep erythrocytes and guinea pig complement. Intact, virus-specific antibody reacted with the antigen on the surface of the erythrocytes, fixed complement, and produced a ring of hemolysis. The area of the hemolysis was proportional to the concentration of Fc functional antibody for that specific antigen.

Viral Antigens. Rubella antigen was obtained from Whittaker Bioproducts, Inc. (Walkersville, Md., USA). Influenza A (ATCC VR822) and influenza B (ATCC VR523) viruses isolated from humans (Melbourne, Australia) and throat washings (US patient, 1966), respectively, were propagated in the allantoic cavity of embryonated chicken eggs, and the crude allantoic fluid was used as a source of viral antigen to sensitize periodate-treated sheep erythrocytes (sheep cells in Alsever's solution, Mission Laboratory Supply, Rosemead, Calif., USA).

Preparation of SRH Test Plates. The SRH test plates were prepared from a mixture of 1.5% agarose (Sea-Kem) (International Biotechnologies, Inc., New Haven, Conn., USA) and 1% suspension of viral-antigen-sensitized sheep erythrocytes with or without 3.3% guinea pig complement (Gibco, Life Technology Inc., Grand Island, N.Y., USA). Plates were poured at 45°C and allowed to cool. Wells (3 mm) were punched in the plates to contain the test sample.

SRH Test Conditions. Eight microliters of test immunoglobulin preparation was diluted in modified barbital buffer (1.6 m*M* sodium 5,5-diethylbarbiturate, 3.1 m*M* 5,5-diethylbarbituric acid, 0.15 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, and 0.15 *M* NaCl, pH 7.2) and placed in the wells. Plates were incubated at 37°C for 15 h. The plates were then photographed. Control plates free of complement were also prepared. These plates were flooded with complement after the initial incubation period had established that no hemolysis had occurred and incubated for an additional 4–6 h at 37°C. An additional control consisted of Venoglobulin-S (lot GL0005A) digested with pepsin (pepsin-IGIV) for 8 h at 37°C, to demonstrate the requirement for intact Fc function in this assay. Data are presented as reciprocal of the calculated dilution that intercepts with the y-axis.

Opsonophagocytic Killing of Bacterial Pathogens

An opsonophagocytosis assay was used to determine the activity of the IGIV test samples in mediating opsonophagocytosis of group B *Streptococcus, Staphylococcus aureus,* and *Escherichia coli* by human polymorphonuclear cells (PMNs) in the presence of human complement. Assays were performed using modifications of methods described by others [31–33]. Briefly, the opsonophagocytic killing assay was performed in a final reaction mixture (100 μl) containing 10 μl of diluted test sample, 40 μl of PMNs (2.5×10^7 PMNs/ml, prepared from heparinized whole blood from healthy adults), 10 μl of human cord sera (CS) as a source of complement, 10 μl of bacterial suspension, and 30 μl of Minimal Essential Medium (MEM, Gibco, Detroit, Mich., USA) containing 0.1% gelatin. The bacteria-PMN ratio was approximately 1:5. Controls included reaction mixtures of Venoglobulin, PMN, bacteria, and CS that had been heat-inactivated for 30 min at 56°C (HIΔ56-CS). These assays were performed by S.M. Puentes, MD, Assistant Professor of Medicine, Department of Medicine, Harbor-UCLA Medical Center, Torrance, Calif., USA.

Reaction mixtures were prepared in sterile 12×75 mm polypropylene test tubes, and tumbled at 37°C for 90 min. A 10-µl aliquot was removed from each tube at 0, 45, and 90 min and serially diluted tenfold from 10^{-1} to 10^{-4} to determine bacterial viability. The first dilution was prepared in sterile water to lyse PMNs by hypotonic lysis; the remaining dilutions were prepared in normal saline. Blood agar plates were inoculated with 250 µl of 10^{-2}, 10^{-3}, and 10^{-4} dilutions and incubated overnight at 37°C. The number of bacterial colony-forming units (CFU) was determined, and the percentage of organisms killed was calculated.

Bacteria. Group B *Streptococcus*, Type II, strain 738; *S. aureus*, capsular type 8, strain Gonzalez, and *E. coli*, strain Ec5 [018ac:K1:H7] expressing the K1 antigen (capsular type) are clinical isolates from Harbor-UCLA Medical Center, Torrance, Calif., USA. To prepare an organism for use in the opsonophagocytosis assay, an overnight broth culture was started using a single colony isolated from a blood agar plate. The overnight cultures of group B *Streptococcus* and *S. aureus* were inoculated in Todd-Hewitt broth (THB, Difco), and grown for 90 min at 37°C in a water bath. The overnight culture of *E. coli* was inoculated in Brain Heart Infusion (BHI, Difco) at an overnight broth culture: fresh medium ratio of 1:10 and grown for 60 min at 37°C in a water bath. The organisms were then harvested, washed once in MEM containing 0.1% gelatin, and resuspended to a final concentration of 2×10^7 organisms/ml.

Analysis of IgG Aggregates

Three methods were used to detect immune complexes and/or aggregated immunoglobulin in the test samples. Enzyme immunoassays (EIAs) using $F(ab')_2$ anti-C3 ($F(ab')_2$ anti-C3 EIA) and Raji cells (Raji-EIA) were performed according to published methods [34]. A micromodification of a previously described C1q-EIA [35] was also used. These assay systems make it possible to evaluate the content of immune complexes and aggregated immunoglobulin in unmodified IGIV products. In addition, if the antibodies retain Fc function, high binding occurs in heat-aggregated samples. Test samples included appropriate dilutions of unmodified and heat-aggregated Venoglobulin (63°C for 15 min). Test samples were compared to known levels of aggregated human globulin (AHG). Assay conditions are described briefly below. These studies were performed by S.C. Jordan, MD, and M. Toyoda, PhD, of Pediatric Nephrology and Transplant Immunology at Cedars-Sinai Medical Center, Los Angeles, Calif., USA.

$F(ab')_2$ anti-C3 EIA. Each well of a microtier plate was coated with 100 µl of goat $F(ab')_2$ antihuman C3 (100 µg/ml) at 37°C for 1 h and overnight at 4°C. The wells were washed with phosphate-buffered saline (PBS)-Tween 20 (0.05%) three times. The test sample was diluted with PBS containing normal human serum as complement source; 100 µl of the diluted sample was added to each well. The plate was incubated (37°C, 2 h), and the wells were washed with PBS-Tween 20. Horseradish peroxidase (HRP)-conjugated protein A (100 µl) was added to each well, and the plate was incubated at 37°C for 1 h, then washed four times. The enzymatic activity remaining in each well was determined by adding 100 µl of *o*-phenylenediamine (OPD) in citrate buffer containing H_2O_2; after several minutes, the reaction was stopped by adding 50 µl of 4.5 *N* H_2SO_4, and the absorbance at 490 nm was measured with an automated spectrophotometer.

Raji-EIA. Raji cell suspension, 50 µl (2×10^5), and 20 µl of test solution were added to each well and incubated for 1 h at 37°C. The cells were washed three times with MEM, 50 µl of HRP-protein-A conjugate was added to each well, and the plates were incubated for 1 h at 37°C. After washing, 50 µl of OPD/H_2O_2 was added to each well. The reaction was stopped after several minutes by the addition of 50 µl of 4.5 *N* H_2SO_4, and the absorbance at 490 nm was measured with an automated spectrophotometer.

C1q-EIA. Microtiter plates were coated with C1q as described for $F(ab')_2$ anti-C3. All other assay conditions were similar to those described for the $F(ab')_2$ anti-C3 EIA, except that the incubation with HRP-protein-A conjugate was carried out for an additional 30 min at 4°C.

Gel Filtration (HPLC)

Analysis of the relative amount of IgG aggregates and monomer content was done by high-performance liquid chromatography (HPLC). A 7.5-cm GSWP column was used as a precolumn and a 60-cm G3000SW column was used as the fractionation column (Toyo Soda Manufacturing, Tokyo, Japan). Both the GSWP and the G3000SW columns were equilibrated with 0.05 *M* acetate buffer containing 0.15 *M* NaCl, pH 6.6; 20 µl of a sample solution was passed through the columns at a flow rate of 0.5 ml/min. Protein concentration was monitored by absorbance at 280 nm.

Results

Single Radial Hemolysis Assay

The area of hemolysis was determined by measuring the diameter of the ring for Venoglobulin-S, Venoglobulin-I, and cryoprecipitate-poor human plasma pool (each diluted 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160) and using this measurement to calculate the area in square millimeters. Each of the samples produced similar reaction patterns at comparable dilutions (data not shown). Because the area of the hemolyzed region for each sample is proportional to the logarithm of the antibody concentration [29], the hemolytic effects of Venoglobulin-S and Venoglobulin-I were analyzed by computing linear regressions for the areas of hemolysis. The y-intercepts from these regression analyses were then compared (table 1). Mean values (± standard error) for the two groups were 179.9±13.0 and 167.5±14.0 with rubella antigen; 163.2±9.9 and 163.1±10.9 with influenza A antigen; and 262.8±6.2 and 253.3±26.1 with influenza B antigen, for Venoglobulin-S and Venoglobulin-I, respectively, showing that there are no differences in hemolytic activity among these preparations.

The hemolytic effects of the test samples were also compared with cryoprecipitate-poor human plasma. The results (table 2) indicated that the paired Venoglobulin-S and Venoglobulin-I samples, which were prepared from the same lot of fraction II paste, showed equivalent Fc-mediated hemolytic function against rubella, influenza A, and influenza B viral antigens. The relative potency of the test samples relative to plasma was found to be approximately sixfold, with a range from 5.0 to 11.01. This corresponds with the IgG concentration in IGIV (50 mg/ml), which is approximately

Table 1. Single radial hemolysis assay for Fc function against viral antigens: relative hemolytic effects

Immunoglobulin preparation	γ-Intercepts for viral antigens		
	rubella	influenza A	influenza B
Pair A			
Venoglobulin-S GL0005A	188.3±10.3	161.4±9.2	255.8±9.0
Venoglobulin-I GV0007A	154.7±8.2	169.1±11.3	259.1±9.4
Pair B			
Venoglobulin-S GL0006A	186.5±11.2	173.9±13.3	265.9±2.6
Venoglobulin-I GV0015A	182.5±6.7	169.7±126.6	276.0±15.2
Pair C			
Venoglobulin-S GL0008A	164.9±9.3	154.3±10.5	266.9±18.6
Venoglobulin-I GV0017A	154.7±14.0	163.1±10.9	253.3±26.1

Relative hemolytic effects of Venoglobulin-S and Venoglobulin-I were analyzed by computing linear regression for the serial dilutions. Shown are the γ-intercepts obtained from these regression analyses ± SEM mean. No hemolysis occurred in the absence of complement.

Table 2. Single radial hemolysis assay for Fc function against viral antigens: hemolytic effect of Venoglobulin lots relative to plasma

Immunoglobulin preparation	Viral antigen		
	rubella	influenza A	influenza B
Pair A			
Venoglobulin-S GL0005A	7.08	6.03	8.34
Venoglobulin-I GV0007A	5.97	6.26	9.74
Pair B			
Venoglobulin-S GL0006A	6.48	5.98	11.01
Venoglobulin-I GV0015A	6.90	6.02	9.27
Pair C			
Venoglobulin-S GL0008A	5.85	5.31	9.13
Venoglobulin-I GV0017A	5.26	5.00	6.07
Cryoprecipitate-poor human plasma pool	1.00	1.00	1.00

Single radial diffusion assays were performed for each viral antigen. The ratio of the dilution of each immunoglobulin preparation was calculated relative to a cryoprecipitate-poor human plasma pool. The plasma data from undiluted to 1:8 and purified immunoglobulin data from 1:5 to 1:40 were used for the calculations.

Table 3. Bacterial killing by opsonophagocytosis: group B *Streptococcus*

Immunoglobulin preparation	Bacterial killing in the presence of indicated concentration of immunoglobulin preparation (mg/ml), %				
	5.0	2.5	1.25	0.625	0.312
Pair A					
Venoglobulin-S GL0005A	67.2	76.6	68.9	61.7	30.4
Venoglobulin-I GV0007A	79.8	89.3	61.3	35.2	10.3
Pair B					
Venoglobulin-S GL0006A	78.8	80.7	74.6	60.7	20.4
Venoglobulin-I GV0015A	83.2	83.6	56.5	35.4	13.4
Pair C					
Venoglobulin-I GL0008A	73.1	78.6	65.9	47.8	19.5
Venoglobulin-I GV0017A	67.0	63.3	62.3	43.3	2.2

Opsonophagocytosis was performed using PMNs in the presence of immunoglobulin plus human complement. Killing of group B *Streptococcus* by PMNs was determined after 45 min incubation, using the formula below, in which $CFU_{HIA56\text{-}CS}$ indicates the number of CFUs in the presence of heat-inactivated CS, and CFU_{CS} indicates the number of CFUs in the presence of native complement. No bacterial killing occurred in the absence of complement:

$$\%\,\text{killing} = \frac{(CFU_{HIA56\text{-}CS} - CFU_{CS})}{CFU_{HIA56\text{-}CS}} \times 100.$$

five- to eightfold higher than the plasma IgG concentration, therefore comfirming that no molecular damage has taken place during manufacturing.

Opsonophagocytic Killing of Bacterial Pathogens

The opsonophagocytic activity of each Venoglobulin-S and Venoglobulin-I lot was examined at final concentrations of 5.0, 2.5, 1.25, 0.625, and 0.312 mg/ml. The results of the assays were expressed as the average of the percentage of opsonophagocytic killing as shown in tables 3–5, against group B *Streptococcus*, *S. aureus*, and *E. coli*, respectively. When each individual assay result was evaluated, the actual range of killing varied. There appears to be a trend for maximal opsonophagocytic killing to occur at 2.5 mg/ml against group B *Streptococcus* (table 3), at 1.25 mg/ml against *S.*

Table 4. Bacterial killing by opsonophagocytosis: *S. aureus*

Immunoglobulin preparation	Bacterial killing in the presence of indicated concentration of immunoglobulin preparation (mg/ml), %				
	5.0	2.5	1.25	0.625	0.312
Pair A					
Venoglobulin-S GL0005A	72.3	70.5	71.2	66.9	71.1
Venoglobulin-I GV0007A	66.3	72.4	71.3	73.9	73.7
Pair B					
Venoglobulin-S GL0006A	66.5	70.1	72.7	65.7	64.1
Venoglobulin-I GV0015A	72.2	56.8	66.0	71.1	79.8
Pair C					
Venoglobulin-I GL0008A	66.9	69.4	75.5	65.1	66.8
Venoglobulin-I GV0017A	68.8	69.0	72.2	74.5	67.2

Opsonophagocytosis was performed using PMNs in the presence of immunoglobulin plus human complement. Killing of *S. aureus* by PMNs was determined after 90 min incubation, using the formula below, in which $CFU_{0\ min}$ indicates the number of CFUs in the initial reaction mixture, and $CFU_{90\ min}$ indicates the number of CFUs present after 90 min incubation. No bacterial killing occurred in the absence of complement:

$$\%\ killing = \frac{(CFU_{0\ min} - CFU_{90\ min})}{CFU_{0\ min}} \times 100.$$

Table 5. Bacterial killing by opsonophagocytosis: *E. coli*

Immunoglobulin preparation	Bacterial killing in the presence of indicated concentration of immunoglobulin preparation (mg/ml), %				
	5.0	2.5	1.25	0.625	0.312
Pair A					
Venoglobulin-S GL0005A	24.6	32.4	35.6	20.9	7.6
Venoglobulin-I GV0007A	9.1	18.0	16.5	8.8	0
Pair B					
Venoglobulin-S GL0006A	44.3	31.1	30.8	0	1.0
Venoglobulin-I GV0015A	20.8	27.9	18.9	17.6	8.6
Pair C					
Venoglobulin-S GL0008A	33.6	26.7	21.9	8.9	18.2
Venoglobulin-I GV0017A	30.7	9.3	28.2	9.8	7.9

Opsonophagocytosis was performed using PMNs in the presence of immunoglobulin plus human complement. Killing of *E. coli* by PMNs was determined after 45 min incubation using the formula below, in which $CFU_{HIA56-CS}$ indicates the number of CFUs in the presence of heat-inactivated CS, and CFU_{CS} indicates the number of CFUs in the presence of native complement. No bacterial killing occurred in the absence of complement:

$$\%\ killing = \frac{(CFU_{HIA56-CS} - CFU_{CS})}{CFU_{HIA56-CS}} \times 100.$$

aureus (table 4), and at 5.0 mg/ml against *E. coli* (table 5). No killing of bacterial pathogens occurred in the absence of complement.

IgG Aggregate Levels

Analysis by C1q-EIA (fig. 1) showed that both Venoglobulin preparations had very low C1q binding, which is known to bind to aggregated IgG. This is demonstrated in the heat-aggregated samples, which showed an 18-fold increase for Venoglobulin-S (to 2,828–5,500 µg AHG Eq/ml) and five-fold for Venoglobulin-I (to 1,797–4,044 µg AHG Eq/ml). In the F(ab')$_2$-anti-C3-EIA assay, levels were again increased in heat-aggregated samples: 18-fold for Venoglobulin-S (ranging from 7,228–10,256 µg AHG Eq/ml to 130,700–174,120 µg AHG Eq/ml) and 10-fold for Venoglobulin-I (ranging from 11,000–15,744 µg AHG Eq/ml to 116,440–128,360 µg AHG Eq/ml) (fig. 2). The Raji-EIA showed low levels of immune complexes for all unheated samples (fig. 3); again, heat aggregation increased the levels by 17-fold for Venoglobulin-S and 10-fold for Venoglobulin-I. HPLC also showed the highly monomeric nature of the products, with monomers ranging between 87 and 90%, dimers between 9 and 12%, and polymers less than 0.3% (table 6).

Table 6. HPLC analysis of IGIV preparations

Immunoglobulin preparation	IgG, %		
	polymer	dimer	monomer
Pair A			
Venoglobulin-S GL0005A	≤0.3[1]	10.6	89.2
Venoglobulin-I GV0007A	0.6	10.1	89.2
Pair B			
Venoglobulin-S GL0006A	≤0.3	10.0	89.8
Venoglobulin-I GV0015A	0.8	11.8	87.4
Pair C			
Venoglobulin-S GL0008A	≤0.3	9.4	90.3
Venoglobulin-I GV0017A	0.8	11.5	87.6

Relative amount of IgG aggregates and monomer content was done by HPLC.

[1] Detection limit.

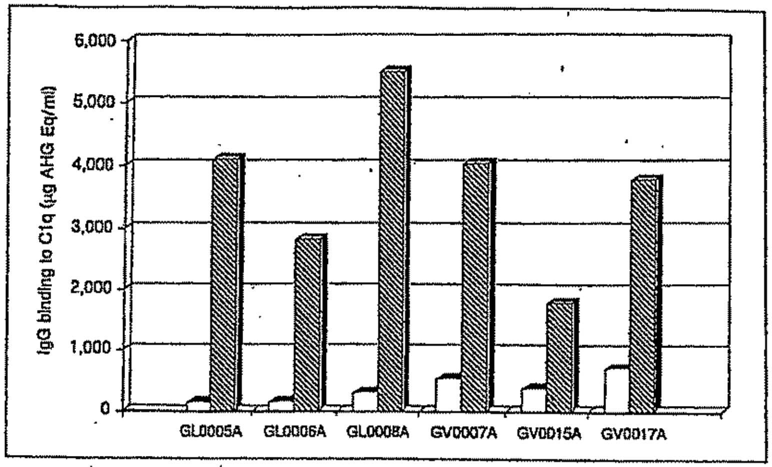

Fig. 1. IgG binding to C1q in unmodified (□) and heat-aggregated (▨) Venoglobulin preparations measured by C1q-EIA. Results are expressed in terms of μg AHG Eq/ml.

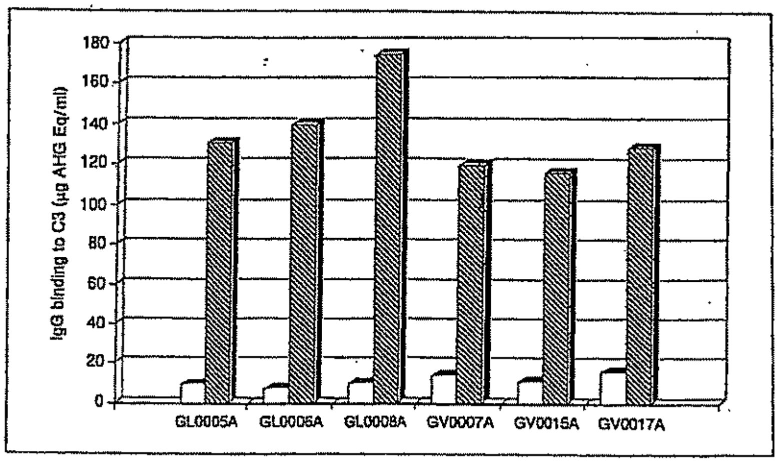

Fig. 2. IgG binding to C3 in unmodified (□) and heat-aggregated (▨) Venoglobulin preparations measured by $F(ab')_2$-anti-C3-EIA. Results are expressed in terms of μg AHG Eq/ml.

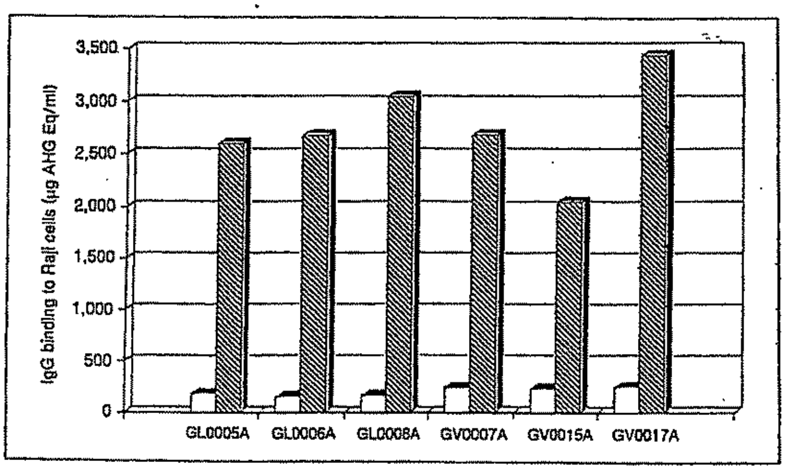

Fig. 3. IgG binding to Raji cells in unmodified (□) and heat-aggregated (▨) Venoglobulin preparations measured by Raji-EIA. Results are expressed in terms of μg AHG Eq/ml.

Discussion

The search for immunoglobulin preparations suitable for intravenous administration has been characterized by attempts to increase safety while retaining essential effector functions of the infused antibody molecules [36, 37]. Early attempts to provide intravenous therapy with immunoglobulin were hampered by severe adverse reactions that were attributed to aggregated immunoglobulin. Enzymatic degradation was used to circumvent this problem. However, the short half-life of the resulting preparations rendered them unsuitable for long-term use as antibody replacement therapy. Thus, efforts were directed at developing immunoglobulin preparations that would retain the biological properties of native immunoglobulin, and in 1984, Pirofsky [36] reported the successful treatment of 30 patients with primary immunodeficiency disease with the first-generation IGIV product (nonintact) 'prepared by mild disulfide reduction and alkylation of serum immune globulin'. In 1985, Korninger et al. [38] reported that a second-generation IGIV product (intact) was effective in treating severe chronic idiopathic thrombocytopenic purpura in adults, whereas a pepsin-treated preparation (nonintact) was ineffective.

Recently, the transmission of a number of human pathogenic viruses by plasma derivatives has caused the safety question to be readdressed, and Venoglobulin-S, the first third-generation IGIV product (intact), which contains a deliberate virus inactivation step, was licensed in the United States in November 1991. We have reported that this method is effective in inactivating and/or eliminating a wide range of experimental marker viruses and human pathogenic viruses [39].

Understanding of the complex mechanisms by which IGIV exerts its therapeutic effects in a variety of diseases has advanced appreciably since the initial attempts at IGIV therapy. Because multiple factors are believed to be required for full biological activity, Kwang Sik Kim, MD, proposed that 'To ensure that preparations are therapeutic, their antibody levels must be assessed both by antigen-binding assays, such as enzyme-linked immunosorbent assay (ELISA) and functional assays such as opsonic (in vitro) and protective (in vivo) activities' [2].

EIAs have confirmed that Venoglobulin-S preparations contain antibodies directed at a wide range of human pathogens, including viral, bacterial, mycoplasmal, and fungal antigens (data not shown). In addition, using a virus-neutralizing antibody assay [40], Venoglobulin-S and Venoglobulin-I were found to contain comparable amounts of cytomegalovirus-neutralizing antibodies (68 Paul-Ehrlich units [PEU]/ml and 59 PEU/ml, respectively [41]).

In the present study, a new manufacturing process for the preparation of virally inactivated IGIV was evaluated with respect to the biological activity of the resulting IGIV. Paired samples were prepared from three different plasma lots, and compared by in vitro tests that measure biological activities dependent on intact Fc function. Complement-dependent hemolysis was measured in a SRH test to determine activity against viral antigens (rubella, influenza A, and influenza B). Both formulations showed equivalent Fc function by the single radial hemolysis test.

The opsonophagocytosis assays reported here are based on an assay system reported by Hill et al. [31]. These investigators demonstrated that complement was required for optimum killing of group B *Streptococci*, *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli*. Moreover, in comparing two IGIV preparations, these investigators found that modifications in the production technique markedly affected the biological activity of the resulting product [31]. In the present study, opsonophagocytic killing of bacterial pathogens (group B *Streptococcus*, *S. aureus*, and *E. coli*) in the presence of antibody, normal human PMNs, and human complement was measured. Both formulations expressed opsonophagocytic killing against these organisms at IGIV concentrations as low as 0.3 mg/ml. Complement binding of native and heat-aggregated IGIV was measured using a C1q-protein-A EIA, an $F(ab')_2$-anti-C3-EIA, and a Raji cell EIA. Complement binding was low in the native IGIV preparations, and enhanced after heat aggregation, indicating the monomeric nature of the native product. Thus, the solvent-detergent-treated IGIV product retains a high level of Fc function.

Conclusions

In SRH assays, paired preparations of Venoglobulin-S and Venoglobulin-I showed equivalent function against rubella, influenza A, and influenza B viral antigens in the presence of complement. Paired lots of Venoglobulin-S and Venoglobulin-I possessed opsonophagocytic killing activity against all bacterial pathogens tested. Immune complex/aggregate levels in three preparations of Venoglobulin-S and Venoglobulin-I were low; enhanced complement-binding activity of the heat-aggregated preparations confirmed that these preparations retain the potential to fix complement. Thus, as shown by these in vitro assays, the third-generation, virus-inactivated immunoglobulin product retains the Fc functions believed to be required for full clinical activity.

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Form PTO 948 (Rev. 10-94) U.S. DEPARTMENT OF COMMERCE - Patent and Trademark Office Application No. 532211

NOTICE OF DRAFTSPERSON'S PATENT DRAWING REVIEW

PTO Draftpersons review all originally filed drawings regardless of whether they are designated as formal or informal. Additionally, patent Examiners will review the drawings for compliance with the regulations. Direct telephone inquiries concerning this review to the Drawing Review Branch, 703-305-8404.

The drawings filed (insert date) 9/22/95, are
A. ✓ not objected to by the Draftsperson under 37 CFR 1.84 or 1.152.
B.____ objected to by the Draftsperson under 37 CFR 1.84 or 1.152 as indicated below. The Examiner will require submission of new, corrected drawings when necessary. Corrected drawings must be submitted according to the instructions on the back of this Notice.

1. DRAWINGS. 37 CFR 1.84(a): Acceptable categories of drawings: Black ink. Color.
   - ___ Not black solid lines. Fig(s)____
   - ___ Color drawings are not acceptable until petition is granted. Fig(s)____
2. PHOTOGRAPHS. 37 CFR 1.84(b)
   - ___ Photographs are not acceptable until petition is granted. Fig(s)____
   - ___ Photographs not properly mounted (must use brystol board or photographic double-weight paper). Fig(s)____
   - ___ Poor quality (half-tone). Fig(s)____
3. GRAPHIC FORMS. 37 CFR 1.84 (d)
   - ___ Chemical or mathematical formula not labeled as separate figure. Fig(s)____
   - ___ Group of waveforms not presented as a single figure, using common vertical axis with time extending along horizontal axis. Fig(s)____
   - ___ Individuals waveform not identified with a separate letter designation adjacent to the vertical axis. Fig(s)____
4. TYPE OF PAPER. 37 CFR 1.84(e)
   - ___ Paper not flexible, strong, white, smooth, nonshiny, and durable. Sheet(s)____
   - ___ Erasures, alterations, overwritings, interlineations, cracks, creases, and folds copy machine marks not accepted. Fig(s)____
   - ___ Mylar, velum paper is not acceptable (too thin). Fig(s)____
5. SIZE OF PAPER. 37 CFR 1.84(f): Acceptable sizes:
   - 21.6 cm. by 35.6 cm. (8 1/2 by 14 inches)
   - 21.6 cm. by 33.1 cm. (8 1/2 by 13 inches)
   - 21.6 cm. by 27.9 cm. (8 1/2 by 11 inches)
   - 21.0 cm. by 29.7 cm. (DIN size A4)
   - ___ All drawing sheets not the same size. Sheet(s)____
   - ___ Drawing sheet not an acceptable size. Sheet(s)____
6. MARGINS. 37 CFR 1.84(g): Acceptable margins:

Paper size

	21.6 cm. X 35.6 cm. (8 1/2 X 14 inches)	21.6 cm X 33.1 cm. (8 1/2 X 13 inches)	21.6 cm. X 27.9 cm. (8 1/2 X 11 inches)	21.0 cm. X 29.7 cm. (DIN Size A4)
T	5.1 cm. (2")	2.5 cm. (1")	2.5 cm. (1")	2.5cm.
L	.64 cm. (1/4")	.64 cm. (1/4")	.64 cm. (1/4")	2.5 cm.
R	.64 cm. (1/4")	.64 cm. (1/4")	.64 cm. (1/4")	1.5 cm.
B	.64 cm. (1/4")	.64 cm. (1/4")	.64 cm. (1/4")	1.0 cm.

Margins do not conform to chart above.
Sheet(s)____
___Top (T) ___Left (L) ___Right (R) ___Bottom (B)

7. VIEWS. 37 CFR 1.84(h)
   - REMINDER: Specification may require revision to correspond to drawing changes.
   - ___ All views not grouped together. Fig(s)____
   - ___ Views connected by projection lines or lead lines. Fig(s)____
   - Partial views. 37 CFR 1.84(h) 2
   - ___ View and enlarged view not labled separately or properly. Fig(s)____
   - Sectional views. 37 CFR 1.84 (h) 3
   - ___ Hatching not indicated for sectional portions of an object. Fig(s)____
   - ___ Cross section not drawn same as view with parts in cross section with regularly spaced parallel oblique strokes. Fig(s)____
8. ARRANGEMENT OF VIEWS. 37 CFR 1.84(i)
   - ___ Words do not appear on a horizontal, left-to-right fashion when page is either upright or turned so that the top becomes the right side, except for graphs. Fig(s)____
9. SCALE. 37 CFR 1.84(k)
   - ___ Scale not large enough to show mechanism with crowding when drawing is reduced in size to two-thirds in reproduction. Fig(s)____
   - ___ Indication such as "actual size" or scale 1/2" not permitted. Fig(s)____
10. CHARACTER OF LINES, NUMBERS, & LETTERS. 37 CFR 1.84(l)
    - ___ Lines, numbers & letters not uniformly thick and well defined, clean, durable, and black (except for color drawings). Fig(s)____
11. SHADING. 37 CFR 1.84(m)
    - ___ Solid black shading areas not permitted. Fig(s)____
    - ___ Shade lines, pale, rough and blurred. Fig(s)____
12. NUMBERS, LETTERS, & REFERENCE CHARACTERS. 37 CFR 1.84(p)
    - ___ Numbers and reference characters not plain and legible. 37 CFR 1.84(p)(1) Fig(s)____
    - ___ Numbers and reference characters not oriented in same direction as the view. 37 CFR 1.84(p)(1) Fig(s)____
    - ___ English alphabet not used. 37 CFR 1.84(p)(2) Fig(s)____
    - ___ Numbers, letters, and reference characters do not measure at least .32 cm. (1/8 inch) in height. 37 CFR(p)(3) Fig(s)____
13. LEAD LINES. 37 CFR 1.84(q)
    - ___ Lead lines cross each other. Fig(s)____
    - ___ Lead lines missing. Fig(s)____
14. NUMBERING OF SHEETS OF DRAWINGS. 37 CFR 1.84(t)
    - ___ Sheets not numbered consecutively, and in Arabic numerals, beginning with number 1. Sheet(s)____
15. NUMBER OF VIEWS. 37 CFR 1.84(u)
    - ___ Views not numbered consecutively, and in Arabic numerals, beginning with number 1. Fig(s)____
    - ___ View numbers not preceded by the abbreviation Fig. Fig(s)____
16. CORRECTIONS. 37 CFR 1.84(w)
    - ___ Corrections not made from prior PTO-948. Fig(s)____
17. DESIGN DRAWING. 37 CFR 1.152
    - ___ Surface shading shown not appropriate. Fig(s)____
    - ___ Solid black shading not used for color contrast. Fig(s)____

COMMENTS:

ATTACHMENT TO PAPER NO. 2 REVIEWER JDC DATE 11/15/95

PTO Copy

REMINDER

Drawing changes may also require changes in the specification, e.g., if Fig. 1 is changed to Fig. 1A, Fig. 1B, Fig. 1C, etc., the specification, at the Brief Description of the Drawings, must likewise be changed. Please make such changes by 37 CFR 1.312 Amendment at the time of submitting drawing changes.

INFORMATION ON HOW TO EFFECT DRAWING CHANGES

1. Correction of Informalities–37 CFR 1.85

File new drawings with the changes incorporated therein. The application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application, should be placed on the back of each sheet of drawings in accordance with 37 CFR 1.84(c). Applicant may delay filing of the new drawings until receipt of the Notice of Allowability (PTOL-37). Extensions of time may be obtained under the provisions of 37 CFR 1.136. The drawing should be filed as a separate paper with a transmittal letter addressed to the Drawing Review Branch.

2. Timing of Corrections

Applicant is required to submit acceptable corrected drawings within the three-month shortened statutory period set in the Notice of Allowability (PTOL-37). If a correction is determined to be unacceptable by the Office, applicant must arrange to have acceptable correction resubmitted within the original three-month period to avoid the necessity of obtaining an extension of time and paying the extension fee. Therefore, applicant should file corrected drawings as soon as possible.

Failure to take corrective action within set (or extended) period will result in ABANDONMENT of the Application.

3. Corrections other than Informalities Noted by the Drawing Review Branch on the Form PTO 948

All changes to the drawings, other than informalities noted by the Drawing Review Branch, MUST be approved by the examiner before the application will be allowed. No changes will be permitted to be made, other than correction of informalities, unless the examiner has approved the proposed changes.

1806

PATENT
MSB-7232

#3/A
10-12-96

I, Luajuana Riley, do hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail on the date indicated below and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20231.

Luajuana Riley
Luajuana Riley

May 9, 1996
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: WILLIAM R. ALONSO	RESPONSE
Serial No.: 08/532,211	PRIMARY EXAMINER: T. R. Scheiner
Filed: September 22, 1995	
Title: PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN	ART UNIT: 1800

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

This is a response to the Official Action mailed February 9, 1996. Please enter the following amendment and remarks.

IN THE CLAIMS:

In claim 1, step (a), line 3, insert --increased-- before "level".

REMARKS

Basis for adding the word --increased-- to step (a) in sole independent claim 1 can be found on page 11 in the first line after Table 1.

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RE THE REJECTIONS UNDER 35 USC 112: The expression "given level" of anticomplementary activity (ACA) has been modified to require that it be a "given increased level". It is submitted that the earlier bases for rejection under 112 on the grounds that the language is vague should be reconsidered and found inapplicable. The increased level is demonstrated in the Examples and Figure.

The expression "acceptable level" of ACA appearing in claim 1 satisfies the requirements of 35 USC 112 and is not vague language. The acceptable level of ACA generally depends on IGIV concentration and examples (for 5 and 10% IGIV solutions) are described in the second full paragraph of page 9.

RE THE REJECTION UNDER 35 USC 103: As may be appreciated by the Examiner, the origin of the invention is the discovery by the applicant that using the trialkyphosphate/detergent viral inactivation method of Neurath et al. for an immune globulin preparation resulted in a surprising but undesirable increase in ACA. To treat the immune globulin preparation in a manner that assures substantial reduction of viral activity (as defined in the application) the conditions of the treatment of step (a) results in an increased ACA level. This increase is now a requirement in step (a) of the claimed methods.

In step (b), the inventor requires that the product of step (a) be incubated under conditions sufficient to bring about a decrease in ACA to an acceptable level.

The Examiner appears to take the position that a combination of the solvent/detergent treatment of Neurath et al. when combined with the immune globulin preparation of Tenold would suggest the method claims under 35 USC 103. Applicants disagree.

The invention can be illustrated better perhaps by viewing the enclosed modified copy of the figure filed with this application. The only modification to the figure is the insertion of the value of ACA level in the control sample prior (corresponding to Tenold). See Table 1, page 11. This is shown to the left of the TNBP treatment represented by the center bar. Thus, in looking at the enclosed revised figure one can see that the original level of ACA in the control must be first increased by the TNBP treatment of step (a) followed by a decrease caused by the incubation requirements of step (b).

There is no suggestion in the prior art, alone or combined, that shows or suggests an increase in ACA level followed a decrease in ACA level to result in a viral inactivated immune globulin preparation with an acceptable ACA level. Thus, the main basis for rejection under 35 USC 102 (Tenold in view of Neurath et al.) should be reconsidered as no longer applicable.

OTHER REFERENCES: Since the Mitra et al. reference does not show the use of solvent/detergent for viral inactivation and since the Yang et al. article does not show or suggest conditions requiring an increase in ACA level followed by a decrease in ACA level it is submitted that those references would not add to any ground for rejecting the current claims, especially as amended.

In view of the above amendment and remarks, it is submitted that the claims in this application now define patentable subject matter and should be allowed.

PATENT
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If the Examiner responsible for this application has any questions regarding the above amendment or remarks that Examiner is invited to telephone the undersigned at anytime.

Respectfully submitted,

Dated: May 9, 1996

James A. Giblin

James A. Giblin
Attorney for Applicant
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P.O. Box 1986
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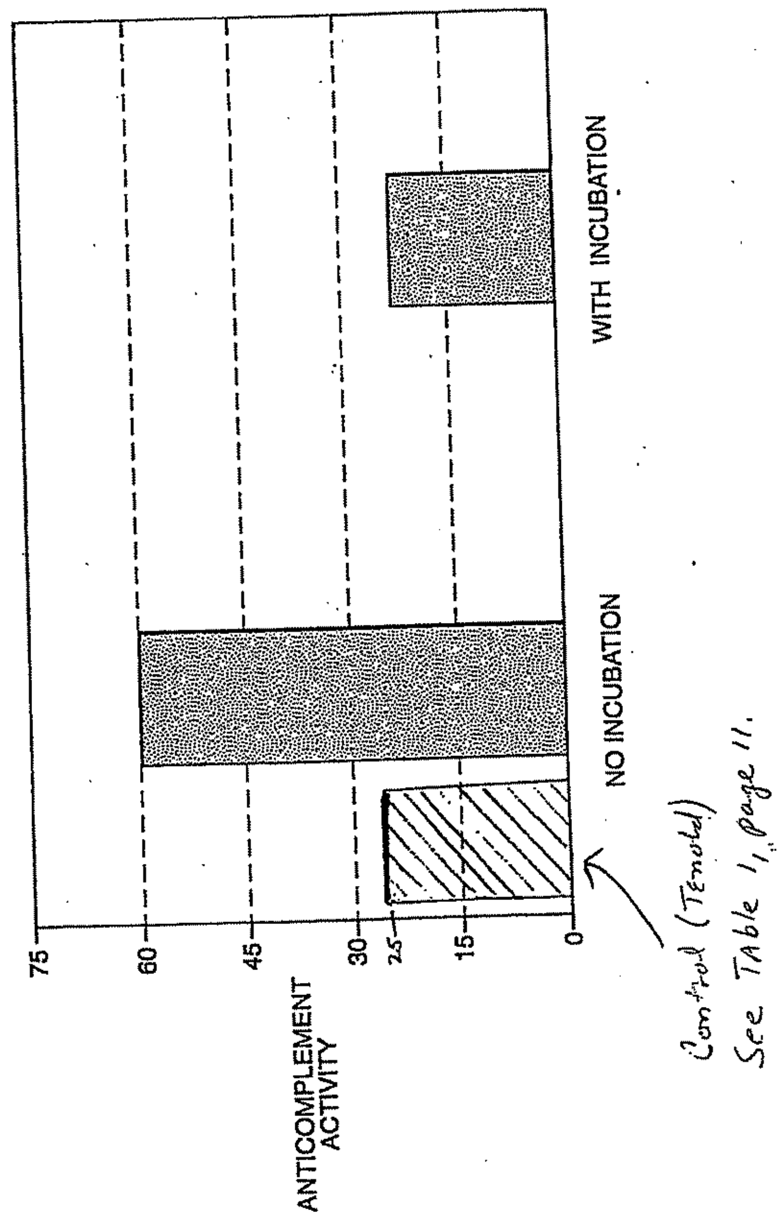
MSB-7232

1 / 1


ANTICOMPLEMENT ACTIVITY
75
60
45
30
25
15
0
NO INCUBATION
WITH INCUBATION
Control (Tenold)
See Table 1, page 11.

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/532,211	09/22/95	ALONSO W	MSB-7232

18M1/0918

JAMES A GIBLIN
BAYER CORPORATION
800 DWIGHT WAY
BERKELEY CA 94701

EYLER, Y	EXAMINER
ART UNIT	PAPER NUMBER
1806	4

DATE MAILED: 09/18/96

Please find below a communication from the EXAMINER in charge of this application.

Commissioner of Patents

TOL-90 (Rev. 6/84)

1 - PATENT APPLICATION FILE COPY

Office Action Summary	Application No. 08/532,211	Applicant(s) Alonso
	Examiner Yvonne Eyler	Group Art Unit 1806

☒ Responsive to communication(s) filed on *May 14, 1996*

☒ This action is FINAL.

☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *3* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *1-24* is/are pending in the application.

Of the above, claim(s) ______ is/are withdrawn from consideration.

☐ Claim(s) ______ is/are allowed.

☒ Claim(s) *1-24* is/are rejected.

☐ Claim(s) ______ is/are objected to.

☐ Claims ______ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on ______ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on ______ is ☐ approved ☐ disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) ______.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: ______

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). ______

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

PAGE 82

— SEE OFFICE ACTION ON THE FOLLOWING PAGES —

Claims 1-24 are pending in the application.

Claim Rejections - 35 USC § 112

1. The rejection of claims 1, 3-6, 10, 21, and 23 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the inventionis maintained.

Claim 1 was rejected as vague and indefinite in the recitations "a given level of anticomplement activity" and "an acceptable level suitable for intravenous administration."

The claim language was amended to recite "a given increased level of anticomplement activity." Further, it was argued that "an acceptable level" is not vague because it depends on the concentration of IGIV.

The latter argument is found to be persuasive, and the rejection based on "an acceptable level suitable for intravenous administration" is withdrawn based on the definition of an acceptable level found in the specification at page 9.

The amended recitation "a given increased level of anticomplement activity" is still found to be vague and indefinite. The use of the term given indicates that the level of anticomplement activity refered to is a precise value which is not included in the claim language, and therefore, it cannot be determined to what the term "given" is referring. Secondly, the incorporation of the term "increased", while more closely

reflecting the invention, does not include a standard against which an increase may be measured.

Claim 10 was found to be vague and indefinite because it recites an "ionic strength less than about 0.001" but fails to define what type of measure or what type of units are associated with the figure 0.001.

Claims 3-6, 21 and 23 refer to a solution comprises either 5% wt/wt antibody or 10% wt/wt antibody. This limitation is vague and indefinite because it is unclear to what the wt/wt refers. If the antibody is in an aqueous solution, then in would be wt/vol. If it is measured as wt/wt, it is unclear what the antibody is being measured with respect to.

Claim Rejections - 35 USC § 103

2. The rejection of claims 1-24 under 35 U.S.C. § 103 as being unpatentable over Tenold (U.S. # 4,396,608) in view of Neurath et al (U.S.# 4,540,573), Mitra et al (U.S.#4,762,714), and Joy Yang et al (Vox Sang 67:337-344, 1994) is maintained.

The claims are drawn to a method of preparing an immune serum globulin solution by first virally inactivating and then incubating under controlled conditions of pH, temperature and tonicity to obtain a low enough level of anticomplement activity to be injected IV.

Applicants argue that the prior art does not teach that the anticomplement activity of the serum preparation actually increases after viral inactivation and must be lowered again by controlled pH, temperature, and tonicity.

While none of the cited art specifically teaches an increase in anticomplement activity after viral inactivation, Neurath et al, Mitra et al, and Joy Yang et al teach the desirabilty of viral inactivation and Neurath et al and Joy Yang et al teach inactivation by solvent/detergent treatment. The prior art in all cases indicates that the level of anticomplement activity must be low for the serum globulin to be injected IV. Tenold et al and Mitra et al teach the reduction of anticomplement activity by incubation under controlled pH, temperature, and tonicity for an extended period of time. Mitra et al teaches to do so after viral inactivation. Thus while the references do not require that the anticomplement activity increase as a result of viral inactivation, such increase subsequently needing to be reduced, it would be obvious to one of skill in the art to monitor anticomplement activity (after viral inactivation or other manipulations) and to treat under conditions of controlled pH, temperature, and tonicity as taught by Tenold et al and Mitra et al to reduce the activity.

No claim is allowed.

3. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a).

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

4. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Yvonne Eyler, Ph.D. whose telephone number is (703) 308-6564. Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Toni R. Scheiner

TONI R. SCHEINER
PRIMARY EXAMINER
GROUP 1800

YE
Yvonne Eyler, Ph.D.
August 15, 1996

MAIL ROOM
NOV 21 1996

PATENT
MSB-7232

I, LuaJuana Riley, do hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail on the date indicated below and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20231.

LuaJuana Riley
LuaJuana Riley
November 18, 1996
Date

5/B
MJS
12/16/96
(NE)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: WILLIAM R. ALONSO

Serial No.: 08/532,211

Filed: September 22, 1995

Title: PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

REQUEST FOR AMENDMENT AFTER FINAL

EXAMINER: Y. EYLER

ART UNIT: 1806

RECEIVED

Commissioner of Patents and Trademarks
Washington, D.C. 20231

OK to enter
12/17/96
& Denny
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Sir:

This is a response to the Final Action mailed September 18, 1996. Please enter the following amendments and the remarks to place this application in condition for allowance or, failing that, in better condition for appeal.

IN THE SPECIFICATION:

At the following places, please insert --M-- after "0.001": Abstract, last line, page 3, line 16, page 3, line 19, page 9, line 7, page 17, line 5.

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IN THE CLAIMS:

In claim 1, step (a), line 3, replace "a given" with --an--.

In claim 1, step (b), line 2, insert the word --increased-- before "anticomplement".

In claim 10, insert --M-- after "0.001".

In claim 21, line 3, insert --M-- after "0.001".

REMARKS

RE: 35 USC 112 (vague and indefinite): The above amendments are requested to overcome the Rejection under 35 USC 112, second paragraph. No new matter is introduced. No new issues are raised. The amendments require that incubation step (b) decreases the amount of anticomplement activity (ACA) caused by step (a). The examiner objected to the use of the word "given". The amendment to step (a) removes that word. Basis for adding the word --increased-- to step (b) can be found in step (a) as amended earlier. With the above amendments the earlier objection to claims 1, 3-6, 10, 21 and 23 as being vague and indefinite should no longer be an issue.

RE: Claim 10 (ionic strength): The examiner found claim 10 vague and indefinite because it recites an ionic strength of less than 0.001 but does not define the units associated with 0.001. Although the applicant believes that the expression of ionic strength in the application as filed is proper, the applicant is requesting that the specification and claims be amended to indicate that the unit associated with ionic strength is molarity.

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At page 9, lines 4-7, the applicants point out that ionic strength was determined in accordance with Perrin (see enclosed copy). Note that the ionic strength described in the Tenold patent, cited by the examiner, also uses the same unit-less method of reflecting ionic strength. See both Perrin and the enclosed copy of page 5 from a standard chemistry text, Biochemical Calculations, 1968, showing how ionic strength is conventionally expressed without units, but the reader of ordinary skill in the art would readily ascertain that the units are molarity.

RE: Claims 3-6, 21 and 23 (use of "wt./wt."): Reconsideration of the rejection of claims 3-6, 21 and 23 is requested. The examiner objected to the designation of "wt./wt." because it was unclear what the wt./wt. referred to.

It is clear from the application and examples that the invention is concerned with treating an aqueous solution of antibodies. Thus, the wt./wt. designation refers to the weight of antibodies (or protein) per unit water. Actual references to the words aqueous or water can be found in the second full paragraph on page 5. See also page 7, seventh line from the bottom. Also, it can be appreciated that the incubation step of (b) for all claims is under controlled conditions of pH and pH by definition is the negative logarithm of hydrogen ion concentration in grams/liter of water. Thus, the reference to either a 5% or 10% wt./wt. designation of the antibody solutions of claims 3-6, 21 and 23 clearly refers to the weight of antibodies per weight of water. The examiner may also refer to Fritz and Schenk, Quantitative Chemistry, p. 8 (copy enclosed) which defines weight per cent concentration as it is known in the art.

REJECTIONS UNDER 35 USC 103: Although it may have been an obvious step to combine the TNBP viral inactivation teachings of Neurath et al. with the immune globulin of Tenold, this would only result in step (a). There is no suggestion or even motivation to take the process one step further by requiring an incubation step (b) since the increase in ACA caused by using TNBP in step (a) was unexpected. A combination of the art as suggested by the examiner assumes the ACA increase was expected but there is no evidence to support that assumption.

Even if it could have been expected, it would not suggest the claims. This is particularly the case in the present invention which shows that the conditions pH, temperature and ionic strength of step (b) must be selected to reduce the ACA to an acceptable level for IV administration. As pointed out on page 9, an acceptable level of ACA will depend on the weight of protein in the solution that is being treated. There is no evidence the prior art even recognized the problem, much less the solution to the problem as described in the claims. Thus, the combination of selected portions of Tenold and Mitra with the viral inactivation of Neurath et al. and Joy Yang et al. requires the use of hindsight. This is clearly impermissible to support a rejection under 35 USC 103.

The examiner is again invited to review the revised Figure included in the earlier response. In the revised Figure (submitted only to illustrate the invention and not for purposes of being a Formal Drawing), the increased ACA observed when using the viral inactivation technique of Neurath et al. (see middle bar) was surprising. It was only by the applicant's discovery of the unexpected increase of ACA caused by step (a) that the follow up invention of step (b) was possible. In other words, if the

immune globulin of Tenold were simply combined with the viral inactivation of Neurath et al., one skilled in the art might not have even expected the rise in ACA, much less discovered a way to reduce it.

The examiner states that Tenold and Mitra et al. teach the reduction of ACA by incubation under controlled pHs, temperature, and tonicity for an extended period of time. Applicants traverse such an interpretation of these references. Tenold teaches a formulation of IgG which yields a preparation already having low ACA which is stable for at least six months. Tenold does not teach how to obtain a decrease in ACA. Moreover, Tenold describes IgG aggregation as causing ACA. In the present invention, lowering of ACA was not due to decreased IgG aggregates because these TNBP/cholate treated IGIV preparations already contained low levels of aggregated IgG (as measured by HPLC) prior to incubation.

Mitra et al. describe the antiviral action of incubation for at least 3 days at pH 4.25, low ionic strength, and 27°C. However, these authors do not disclose a lowering of IgG ACA due to such incubation conditions. Mitra et al. would not have recognized that TNBP treatment caused an increase in ACA and, by teaching a viral inactivation without chemical agents such as TNBP actually teach away from the claims.

In view of the above amendments and remarks, it is submitted that this application is now in condition for allowance and prompt allowance is requested. If the examiner has any further questions regarding the above amendments or remarks, that examiner is invited to telephone the undersigned at anytime.

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Respectfully submitted,

Dated: Nov. 18, 1996

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UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
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Washington, DC 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR		ATTORNEY DOCKET NO.
08/532,211	09/22/95	ALONSO	W	MSB-7232

18M1/1223

JAMES A GIBLIN
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EXAMINER	
EYLER, Y	
ART UNIT	PAPER NUMBER
1806	6

DATE MAILED: 12/23/96

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

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PTO-90C (REV. 2/95)

1-File C

T PRINTING OFFICE 1995-319-826

Advisory Action	Application No. 08/532,211	Applicant(s) Alonso
	Examiner Yvonne Eyler	Group Art Unit 1806

THE PERIOD FOR RESPONSE: [check only a) or b)]

a) ☐ expires ________ months from the mailing date of the final rejection.

b) ☒ expires either three months from the mailing date of the final rejection, or on the mailing date of this Advisory Action, whic is later. In no event, however, will the statutory period for the response expire later than six months from the date of the f rejection.

Any extension of time must be obtained by filing a petition under 37 CFR 1.136(a), the proposed response and the appropriate fee date on which the response, the petition, and the fee have been filed is the date of the response and also the date for the purposes determining the period of extension and the corresponding amount of the fee. Any extension fee pursuant to 37 CFR 1.17 will be calculated from the date of the originally set shortened statutory period for response or as set forth in b) above.

☐ Appellant's Brief is due two months from the date of the Notice of Appeal filed on ____________ (or withi period for response set forth above, whichever is later). See 37 CFR 1.191(d) and 37 CFR 1.192(a).

Applicant's response to the final rejection, filed on *Nov 21, 1996* has been considered with the following effe but is NOT deemed to place the application in condition for allowance:

☒ The proposed amendment(s):

☒ will be entered upon filing of a Notice of Appeal and an Appeal Brief.

☐ will not be entered because:

☐ they raise new issues that would require further consideration and/or search. (See note below).

☐ they raise the issue of new matter. (See note below).

☐ they are not deemed to place the application in better form for appeal by materially reducing or simplifying issues for appeal.

☐ they present additional claims without cancelling a corresponding number of finally rejected claims.

NOTE: ____________

☐ Applicant's response has overcome the following rejection(s):

____

☐ Newly proposed or amended claims ____________ would be allowable if submitte separate, timely filed amendment cancelling the non-allowable claims.

☒ The affidavit, exhibit or request for reconsideration has been considered but does NOT place the application in c for allowance because:
While the intermediate increase in ACA may have been unexpected, the invention of inactivating virus and lowe ACA to acceptable levels would have been obvious to one of ordinary skill in the art, no matter what happened ACA levels in the intermediate steps.

☐ The affidavit or exhibit will NOT be considered because it is not directed SOLELY to issues which were newly ra the Examiner in the final rejection.

☒ For purposes of Appeal, the status of the claims is as follows (see attached written explanation, if any):

Claims allowed: ____________

Claims objected to: ____________

Claims rejected: *1-24*

☐ The proposed drawing correction filed on ____________ ☐ has ☐ has not been approved by the Exan

☐ Note the attached Information Disclosure Statement(s), PTO-1449, Paper No(s). ________.

☐ Other

LILA FEISEE
SUPERVISORY PATENT EXAMINER
GROUP 1800

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U. S. Patent and Trademark Office
PTO-303 (Rev. 8-95) Advisory Action Part of Paper No.

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I, Luajuana Riley, do hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail on the date indicated below and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20231.

Luajuana Riley
Luajuana Riley

February 17, 1997
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: WILLIAM R. ALONSO

Serial No.: 08/532,211

Filed: September 22, 1995

Title: PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN

APPEAL BRIEF

EXAMINER: Y. EYLER

ART UNIT: 1806

RECEIVED
MAR 21 1997
GROUP 1800

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

This is a Brief (3 copies) supporting an appeal from the final rejection mailed July 17, 1996. Authorization is hereby given to charge deposit account 03-4000 the $300.00 Brief Filing Fee under 37 CFR 1.17(f).

(1) REAL PARTY IN INTEREST: The real party in interest is the designated assignee of the application, Bayer Corporation.

(2) RELATED APPEALS AND INTERFERENCES: There are no related Appeals or Interferences regarding the Application.

(3) STATUS OF CLAIMS: Claims 1 through 24, the only claims pending, stand under final rejection. These claims are shown in the attached Appendix.

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I, Luajuana Riley, do hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail on the date indicated below and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20231.

Luajuana Riley
Luajuana Riley

February 17, 1997
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: WILLIAM R. ALONSO	APPEAL BRIEF
Serial No.: 08/532,211	EXAMINER: Y. EYLER
Filed: September 22, 1995	ART UNIT: 1806
Title: PREPARATION OF VIRALLY INACTIVATED INTRAVENOUSLY INJECTABLE IMMUNE SERUM GLOBULIN	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

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(3) STATUS OF CLAIMS: Claims 1 through 24, the only claims pending, stand under final rejection. These claims are shown in the attached Appendix.

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(4) STATUS OF AMENDMENTS: An Amendment after Final Rejection was requested. The Examiner advised that the requested amendments would be entered upon the filing of a Notice of Appeal and this Appeal Brief. That amendment is reflected in the claims of the Appendix.

(5) SUMMARY OF INVENTION: The invention is a method of reducing undesirable anticomplement activity (ACA) resulting from the use of a trialkylphosphate for viral inactivation of a solution of antibodies. The method comprises contacting the solution with the trialkylphosphate under conditions assuring viral inactivation and resulting in an increase in ACA and then incubating the solution under controlled conditions of time, pH, temperature, and ionic strength such that the anticomplement activity is reduced to an acceptable level for intravenous administration of the solution of antibodies. An acceptable ACA level for intravenous administration depends on the antibody concentration. See page 9, lines 14-23 and claims 2-6. The steps of the invention are described in more detail in the Abstract, the Summary of Invention on page 3, lines 8-20 and the claims.

(6) ISSUES: Whether claims 1, 3-6, 10, 21 and 23 should stand rejected under 35 U.S.C. § 112 as vague and indefinite.

Whether claims 1 through 24 should stand rejected under 35 U.S.C. § 103 as unpatentable over Tenold (U.S. 4,396,608) in view of Neurath et al. (U.S. 4,540,573), Mitra et al. (U.S. 4,762,714) and Joy Yang et al. (Vox Sang. 67:337).

(7) GROUPING OF CLAIMS: Claims 1-20 are directed to the method of the invention and stand separate from product by process claims 21-24.

(8) ARGUMENTS:

REJECTIONS UNDER 35 U.S.C. § 112 (second paragraph): Claims 1, 3-6, 10, 21 and 23 were rejected as vague and indefinite in use of the expression, "a given increased level of anticomplement activity". Since the word "given" no longer appears in the claims due to entry of the requested amendment after final, the only issue remaining is whether use of the word "increased" is vague and indefinite on the ground there is no standard against which an increase may be measured. It is respectfully submitted that the "standard" in this application would be the starting ACA level. Since step (a) of the claimed methods results in an increase in ACA from the starting material, a standard is provided. If there is no such increase, then step (b) of the invention, and the invention itself, is not even needed. To illustrate this "standard", the applicant provided in his response of May 9, 1996, a marked up copy of the figure and referred to Table 1 on page 11 to show the "standard" used in that example. A copy of that marked up figure is enclosed with this Brief.

The examiner also objected to the designation of "wt./wt." in claims 3-6, 21, and 23 on the ground it was unclear what the wt./wt. referred to. It is clear from the application and examples that the invention is concerned with treating an aqueous solution of antibodies. Thus, the wt./wt. designation refers to the weight of antibodies (or protein) in a given weight of solution, expressed in a percent basis as is conventional in the art. Basis for an --aqueous-- solution can be found in the second full paragraph on page 5. See also page 7, seventh line from the bottom. Thus, the reference to either a 5% or 10% wt./wt. designation of the antibody solutions of claims 3-6, 21 and 23 clearly refers to the weight of antibodies per weight of solution. See also, Fritz and Schenk, Quantitative Chemistry, p. 8 (copy enclosed) for the definition of weight per cent concentration as it is known in the art.

In view of the amendments and the above arguments, the rejection of claims 1, 3-6, 10, 21 and 23 as being vague and indefinite under 35 USC 112 is improper.

REJECTIONS UNDER 35 USC 103: Although it may have been an obvious step to combine the TNBP viral inactivation teachings of Neurath et al. with the immune globulin of Tenold, this would only result in step (a). There is no suggestion or motivation to take the process one step further by requiring an incubation step (b). The increase in ACA caused by using TNBP in step (a) was unexpected. A combination of the art as suggested by the examiner assumes the ACA increase was expected. There is no evidence to support that assumption.

Even if the ACA increase could have been expected, it would not have suggested the claims. The claimed invention requires that the conditions pH, temperature and ionic strength of step (b) be selected to reduce the ACA to an acceptable level for IV administration. As pointed out on page 9, an acceptable level of ACA will depend on the weight of protein in the solution that is being treated. There is no evidence the prior art even recognized the problem, much less the solution to the problem as described in the claims. Thus, the combination of selected portions of Tenold and Mitra with the viral inactivation of Neurath et al. and Joy Yang et al. requires the use of hindsight. This is clearly impermissible to support a rejection under 35 USC 103.

In the enclosed revised Figure (submitted earlier only to help understand the invention and not for purposes of being a Formal Drawing), the increased ACA observed when using the viral inactivation technique of Neurath et al. (see middle bar) was surprising. It was only by the applicant's discovery of the unexpected increase of ACA caused by step (a) that the follow up of step (b) was possible. In other words, if the immune globulin of Tenold were simply combined with the viral inactivation of Neurath et al., one skilled in the art would not have even expected the rise in ACA, much less discovered a way to reduce it.

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Mitra et al. describe the antiviral action of incubation for at least 3 days at pH 4.25, low ionic strength, and 27°C. However, these authors do not disclose a lowering of IgG ACA due to such incubation conditions. Mitra et al. would not have recognized that TNBP treatment caused an increase in ACA and, by teaching a viral inactivation without chemical agents such as TNBP actually teach away from the claims.

In view of the above remarks, appellants respectfully urge that the rejection of claims 1 through 24 as being obvious under 35 U.S.C. § 103 was improper.

Respectfully submitted,

Dated: February 17, 1997

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